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On page 469, Vol. 132, No. 1, January, 1940, the second sentence should read, *To 1 gm. of 2-methyl-1,4-naphthoquinone were added 2 cc. of glacial acetic acid, 10 cc. of petroleum ether, 1 gm. of zinc dust, and 0 6 cc. of phytyl bromide prepared from phytol*[2] *by the action of phosphorus tribromide.*

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Letters to the Editors

INFLUENCE OF ESTROGEN ON THE ELECTROLYTE PATTERN OF THE IMMATURE RAT UTERUS*

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(Received for publication, October 2, 1939)

The present paper reports measurements on the electrolyte content of immature rat uteri before and after the administration of a single dose of estrogen. It has been shown previously that estrogen causes a prompt and striking increase in the weight and water content of rat uteri and that these changes are followed by a further gain in weight associated with an increase in mitotic activity and protoplasmic growth (1). It is to be expected that there should be shifts in the electrolytes to correspond with the ebb and flow of water. If this thesis is correct, these phenomena should offer an unusual opportunity to study the effect of estrogen on electrolytes and to investigate electrolyte metabolism during normal protoplasmic growth. Furthermore, the pattern of the electrolytes gives some information concerning the location of the water which is responsible for the increase in uterine weight.

The data obtained show that estrogen causes a significant increase in the sodium, chloride, and calcium content of the uterus during the period of edema and later, during the period of protoplasmic growth, an increase in potassium, phosphorus, and magnesium content. At the period of greatest edema (6 hours after estrogen administration), the electrolyte pattern differs considerably from that of untreated controls. The pattern suggests that the edema is both extracellular and intracellular. At the peak of uterine growth (30 hours after estrogen administration), the

* This work was aided in part by a grant from the Rockefeller Foundation administered by Professor F. L. Hisaw.

electrolyte pattern has almost returned to its control configuration.

Materials and Methods

One hundred and eighteen 21 to 22 day-old immature litter mate rats from an inbred colony were used. The average uterine weight of 21 to 26 day-old rats in our colony is 20 mg. and seldom varies more than 2 mg. The uterine weight response to a single dose of estrogen has been observed frequently and found to be consistent. Rats were killed with chloroform before and at varying intervals after the administration of 0.1 microgram of α-estradiol in 0.1 cc. of sesame oil subcutaneously. The uteri were removed immediately, blotted on filter paper, dissected free from adjacent tissues, and placed in tightly stoppered tubes. The uteri of several rats were usually collected in one tube for analysis, but in one series of twenty-four rats, the uteri were collected separately and analyzed individually.[1] In Group A (Table I) a sample of pooled blood was obtained with a dry syringe during life and the serum analyzed. The hearts of the same group were removed at autopsy, pooled in a test-tube after being blotted carefully, and analyzed.

The methods employed will be described in detail by one of us (O. H. L.) elsewhere.[2] The fat-free solid weight was obtained according to the directions of Hastings and Eichelberger (2). Chloride was determined by Volhard titration, sodium as sodium zinc uranyl acetate, and potassium as potassium chloroplatinate gravimetrically. Calcium was precipitated as the oxalate and titrated after conversion to carbonate. Magnesium was precipitated as magnesium ammonium phosphate and the phosphate determined. The total phosphorus was determined colorimetrically with the Fiske and Subbarow (3) reagents. A single sample of about 0.3 gm. of tissue representing ten to twenty ut[illegible] was used for the determinations on pooled samples. On [illegible] amount of tissue the error of the methods is estimated at [illegible] per cent for Cl and P, ±3 per cent for Mg, 0 to 5 per cent K and Na, and ±5 per cent for Ca.

[1] Four groups of animals were studied at different times. These [illegible] presented as Groups A, B, C, and D in Table I. The measurements individual rats are shown in Fig. 2.

[2] Lowry, O. H., unpublished data.

TABLE I

·ytes of Immature Rat Uteri, Hearts, and Serum before and after Administration of Single Dose of α-Estradiol

Period	Material	Group	No. in group	Average weight, fat-free	Average H_2O per uterus	Average solids per uterus	Amount per kilo fat-free tissue: H_2O	Fat-free solids	Cl	P	Na	K	Ca	Mg	K:P	Extracellular H_2O per kilo fat-free tissue*: From Cl	From Na	Intracellular H_2O per kilo cells†: From Cl	From Na	K per kilo fat-free solids
				mg.	mg.	mg.	gm.	gm.	m.eq.	mM	m.eq.	m.eq.	m.eq.	m.eq.		gm.	gm.	gm.	gm.	m.eq.
Control	Pooled uteri	A	20	16	13.2	2.83	823	176.9	62.3	86.7	63.0	96.9			1.12	536	439	629	692	548
	" "	B	20	19.6	16.4	3.24	835	165.0	58.9	92.8	53.7	94.7	3.34	13.7	1.02	512	370	672	744	574
				17.8	**14.8**	**3.04**	**830**	**171**	**60.6**	**89.7**	**58.3**	**95.8**	**3.34**	**13.7**	**1.07**	**524**	**405**	**652**	**718**	**560**
	Hearts	A	20				798.2	201.8	28.9							249		733		
	Serum	"					936	64.0	104.3		139.7									
6 hrs.	Pooled uteri	"	11	28.1	24.3	3.77	865.7	134.3	69.8			75.7				594	637	685	648	564
	" "	B	14	28.5	24.7	3.69	870.3	129.7	60.7	67.6	76.4	76.7	3.21	11.2	1.13	528	527	735	735	591
	" "	C	9	26.3	22.5	3.75	857.3	142.7	62.8	62.3	73.7	83.9			1.35	546	508	694	717	588
	" "	D	11	28.9	24.8	4.12	857.3	142.7	64.7	72.9	75.6	87.3	3.57	14.1	1.20	562	521	686	712	612
				27.9	**24.0**	**3.83**	**863**	**137**	**64.5**	**67.6**	**75.2**	**80.9**	**3.39**	**12.6**	**1.23**	**558**	**548**	**700**	**703**	**589**
	Hearts	A	11				792.8	207.2	28.9							246		728		
	Serum	"					938	62.0	105.8		146.3									
30 hrs.	Pooled uteri	"	10	39.6	33.6	6.00	848.3	151.7	61.4	76.1	64 9	85.7			1.12	535	454	685	730	564
	" "	B	9	35.6	30.1	5.40	844.6	155.4	59.6	87.2	56.8	94.3	4.17	10.9	1.07	518	392	688	752	606
	" "	D	10	34.9	29.2	5.63	838.3	161.7	60.1	84.4	66.7	89.1	3.32	14.9	1.04	522	460	672	708	550
				36.7	**31.0**	**5.68**	**843.7**	**156.3**	**60.4**	**82.6**	**62.8**	**89.7**	**3.75**	**12.9**	**1.08**	**525**	**435**	**682**	**730**	**573**
	Hearts	A	10				792.1	207.9	28.4							247		726		
	Serum	"					935.5	64.5	103.1		139.3									

* The extracellular space was estimated by assuming that all the chloride or sodium was extracellular and at a concentration of 115 and 145 milliequivalents per kilo, respectively, except in Group A, in which the concentration was derived from serum data as shown in the text.

† The H_2O per kilo of cells was calculated by subtracting the estimated gm. of extracellular water from the total tissue water and dividing this by the total weight minus the extracellular weight.

Results

Immature Rats. Group A—The data obtained (Table I) show that a single dose of α-estradiol caused definite changes in the

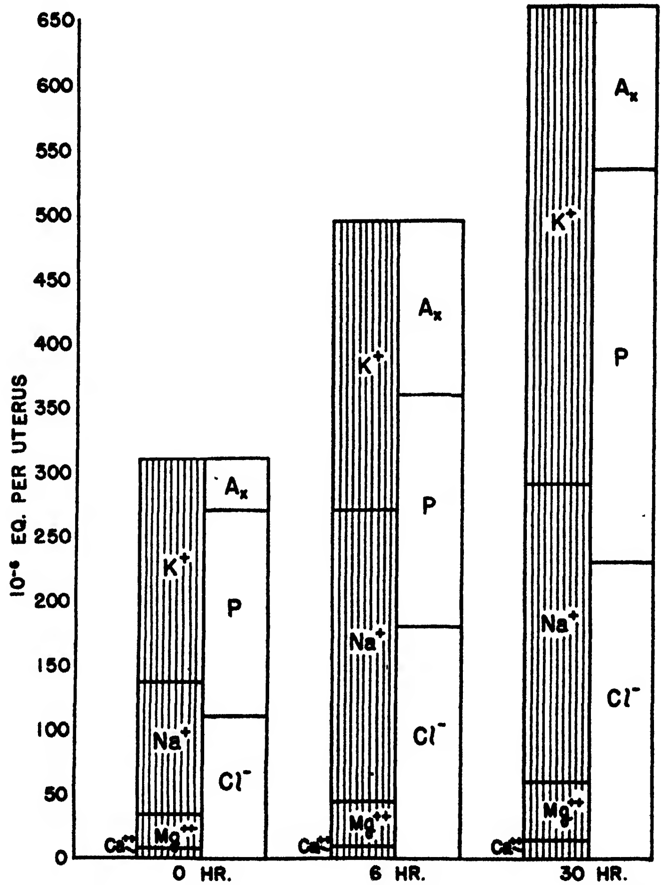

FIG. 1. Average electrolyte content of uteri 0, 6, and 30 hours after the administration of 0.1 microgram of α-estradiol.

electrolyte pattern of the uteri, but not of the hearts or blood serum. All weights are presented on a fat-free basis. The chief features are as follows: (*a*) 6 hours after the administration of α-estradiol, when the uteri were edematous as evidenced by a

decrease in the total solids per kilo, the concentration of sodium had risen significantly and that of chloride slightly, whereas the concentrations of potassium and phosphorus had fallen. Although

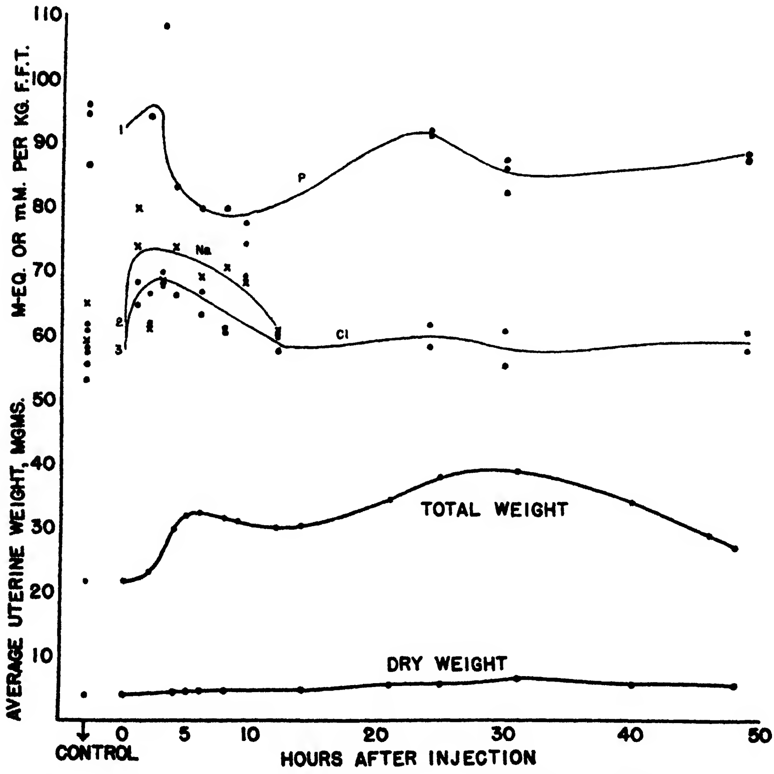

Fig. 2. Observations on the concentrations of electrolytes per kilo of fat-free tissue in individual uteri before and after the administration of 0.1 microgram of α-estradiol. ● = milliequivalents of chloride, ○ = mM of phosphorus, × = milliequivalents of sodium in individual uteri. The fine lines in the upper portion follow the changes in phosphorus (Curve 1), sodium (Curve 2), and chloride (Curve 3), respectively. The two curves in the lower portion show the changes in uterine weight reported previously (1).

the concentration of potassium per kilo of tissue had fallen, the amount of potassium per kilo of total solids had risen as had the ratio of potassium to phosphorus. (*b*) 30 hours after α-estradiol

administration, the uteri were much larger than control uteri but were no longer edematous. These uteri had essentially the same electrolyte pattern per kilo of tissue as did control uteri.

When the average total electrolyte contents of uteri 0, 6, and 30 hours after a single dose of estrogen are calculated (Fig. 1), it becomes evident that there was a striking increase in the electrolyte content of the 6 and 30 hour samples. The cation columns are probably quite accurately representative of the tissue. The anion columns are less accurate, because the valence of the various phosphorus compounds is not known definitely. In Fig. 1 a valence of 1 has been assumed for phosphorus. A_x represents unmeasured anions (HCO_3^-, etc.). At 6 hours there was a relative and absolute increase in A_x.

The electrolyte content per kilo of individual uteri is shown in Fig. 2. That changes in the electrolyte content of the uteri precede changes in water content is the only obvious interpretation of Fig. 2. More data are necessary to establish that point, however.

DISCUSSION

In this discussion sodium and chloride are considered to be chiefly extracellular elements and potassium, magnesium, and phosphorus, intracellular elements. It is recognized, however, that any of these elements may be present to some extent in both phases.

The changes in the electrolyte pattern observed at 6 hours are of interest because they probably precede mitotic activity. At that time the average increase in the uterine water content was 67 per cent. The chloride increase (+63 per cent) and the sodium increase (+116 per cent) were approximately what would be expected if two-thirds of the added water was ultrafiltrate. The increase in potassium and magnesium (+33 per cent) was greater than the increase in solids (+27 per cent). The phosphorus, on the other hand, rose less (+12 per cent) than the solids. The absolute increases in the potassium, phosphorus, and magnesium per uterus without a proportional increase in cell number suggest that there must have been an increase in the cell membrane permeability to these elements. It also seems necessary to consider the possibility of an increase in cell permeability

to sodium and chloride as well. At 30 hours the electrolyte pattern has returned approximately to the control configuration even though the uteri have increased 100 per cent in total weight.

In most tissues the extracellular fluid probably resembles a serum ultrafiltrate. If that is so, the ratio of sodium to chloride (as derived from the Donnan ratio applied to serum values) in the extracellular tissue phase should approximate 1.26. A lower ratio, 0.95, was observed in the control uteri and a ratio of 1.25 in the 6 hour uteri. These findings suggest that at least in the control uteri some of the chloride was intracellular. Manery and Hastings (4) have described other tissues in which there appears to be some intracellular chloride. If some of the chloride is intracellular, chloride cannot be considered an accurate index of the volume of extracellular fluid. On the other hand, because the sodium in the uteri appears to be almost exclusively extracellular, it should serve as an index of the extracellular fluid volume. The data (Table I) support this thesis when the concentration of sodium and chloride in the ultrafiltrate was calculated by assuming a Donnan ratio of 0.95 (2). They were found to approximate 145 and 115 milliequivalents per kilo respectively. On the basis of these values the extracellular volume was calculated and from that the water per kilo of cells was found by subtracting the estimated gm. of extracellular water from the total tissue water and dividing that value by the total weight less the extracellular fluid weight. When this was done, it was found that the chloride values gave cell water contents considerably below 700 gm. per kilo for the control and 30 hour uteri, whereas with a few exceptions the sodium data gave calculated cell water contents that approximated those of the rat hearts and of other striated muscle tissue (2). This is regarded as further evidence that sodium was chiefly extracellular in these uteri and that it therefore should serve as an approximate index of the extracellular fluid content.

Fig. 3 shows the average changes in total solids, and intracellular and extracellular fluid volumes (calculated from sodium) of the uteri 0, 6, and 30 hours after the single dose of estrogen. At 6 hours an increment in intracellular fluid must have been responsible for at least 20 per cent of the total fluid increase. By 30 hours the intracellular fluid has doubled its original volume.

These findings are consistent with the apparent swelling of cells observed histologically (1).

The extent to which the changes observed in the uterus following estrogen stimulation are characteristic of other types of tissue growth remains to be determined. In the case of the uterus the extracellular phase swells 50 to 75 per cent, while the cells increase only one-third as much. During this phase the cells take on their normal quota of potassium but definitely less than their quota of phosphorus and probably of solids. This is followed by a phase characterized by marked cell growth during

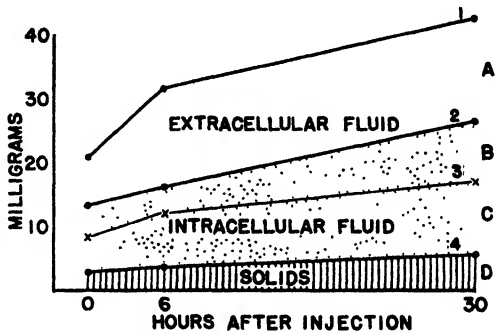

Fig. 3. Changes in average intracellular and extracellular fluid per uterus following administration of 0.1 microgram of α-estradiol, as calculated from chloride and sodium concentrations. The extracellular fluid, as estimated from sodium, is represented by the area *A* which lies between Lines 1 and 2, and as estimated from chloride by the area *A* + *B* which lies between Lines 1 and 3. The intracellular fluid, as calculated from sodium, is represented by the area *B* + *C* between Lines 2 and 4, and, as calculated from chloride, is represented by the area *C* which lies between Lines 3 and 4.

which the cells take up approximately normal amounts of cell constituents.

SUMMARY

An investigation of the electrolyte composition of the immature rat uterus before and after it is stimulated to grow by a single dose of estrogen reveals the following facts. During the first 6 hours after estrogen administration the uterus gains water and chiefly extracellular electrolytes. During this phase there is an increase in the potassium to phosphorus ratio. During

the following 24 hours there is a rapid growth of new protoplasm with a gradual return of the electrolyte pattern to a normal configuration.

We wish to thank Professor A. B. Hastings for his valuable suggestions in the preparation of this paper.

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DEFICIENCY OF VITAMIN B_1 IN MAN AS DETERMINED BY THE BLOOD COCARBOXYLASE

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It is now proved that vitamin B_1 acts in the body as a catalyst, necessary for the degradation of pyruvate, only after it has been converted into its diphosphate ester, cocarboxylase (1). It seems clear that vitamin B_1 circulates in plasma in the free unphosphorylated form; in this form it diffuses readily and passes into tissue fluid, cerebrospinal fluid, urine, and cells of the body. Phosphorylation of the vitamin with formation of cocarboxylase occurs inside the cells (2). We have already indicated that the usefulness for clinical purposes of determinations of cocarboxylase in blood might be limited because all the cocarboxylase in blood is intracellular (2). But since the amount of free vitamin B_1 in plasma is so small (about 1 microgram per 100 ml.) and is considerably affected by slight hemolysis of the blood, no method of sufficient sensitivity and accuracy is available for estimations on plasma. In this paper we propose to examine further the value for clinical purposes of determinations of cocarboxylase in blood by analyzing the results of such estimations in groups of individuals with various diseases, and by comparing these values with the values for the total vitamin B_1 (free and phosphorylated) in blood.

To determine cocarboxylase we have used the modification of the method of Ochoa and Peters that we recently described (2). Total vitamin B_1 has been estimated by applying a correction (shortly to be published by Sinclair) to the values obtained by a slight modification of Meiklejohn's method (3-4). Each of the samples of blood examined was obtained from a hospital patient. We have elsewhere reported values on normal healthy adults (2).

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Comparison of Values for Cocarboxylase with Corresponding Values for Total Vitamin B_1—Simultaneous estimations of cocarboxylase and total vitamin B_1 were made on 80 different samples of blood. A statistical analysis of the results in 77 of these showed a definite correlation between the two values. The cocarboxylase values ranged from 0.0 to 13.0 micrograms per 100 ml. with a mean of 4.14; the values for total vitamin B_1 ranged from 1.5 to 13.0 micrograms per 100 ml. with a mean of 5.11. The correlation coefficient was 0.76 ± 0.05, t (5) = 15.2. Of the three samples omitted from this analysis, two came from patients who had received crystalline vitamin B_1 parenterally within 24 hours of the collection of the blood. In one of these the value for cocarboxylase was 2.5 micrograms per 100 ml. and the total vitamin B_1 13.0; the corresponding values in the other were 5.0 and 12.0 micrograms. The third sample omitted from the analysis was taken from a patient with myeloid leucemia who had a leucocyte count of 354,000 per microliter; the cocarboxylase value was 42.0 micrograms per 100 ml. and the total vitamin B_1 41.0.

We were able to obtain samples of blood before and after treatment by injection of the vitamin in only eight instances, and the estimations in these are recorded in Table I. Administration of crystalline vitamin B_1 was followed in seven of the eight cases by an increase in both the cocarboxylase and total vitamin B_1 values of the blood. But in one subject the latter value increased markedly after daily injection of 10 mg. of crystalline vitamin B_1 for 1 week, whereas the cocarboxylase value remained unchanged. This patient had chronic glomerular nephritis with marked albuminuria, anasarca, and anemia. It is probable that in this subject the ability to form cocarboxylase was diminished. A similar disability would explain the low cocarboxylase value found together with a normal value for total vitamin in Subjects 2 and 4 (Table I). The two patients with pellagra both had skin lesions that responded to treatment with nicotinic acid; in the first (Subject 8) the peripheral neuritis responded to a full diet with addition of vitamin B_1 and nicotinic acid. The second (Subject 9) is included merely to demonstrate that treatment with nicotinic acid does not affect the method of estimation, as we have already shown (2); it also indicates that pellagra tends to be a multiple deficiency and should therefore not be treated with a single pure vitamin.

TABLE I

Effect of Treatment upon Values for Cocarboxylase and Total Vitamin B_1

Subject No.	Diagnosis	Date	Cocarboxylase	Total vitamin B_1	Treatment	Date	Cocarboxylase	Total vitamin B_1
			γ per 100 ml.	γ per 100 ml.			γ per 100 ml.	γ per 100 ml.
1	Chronic glomerular nephritis	May 25, 1939	3.0	4.0	Crystalline vitamin B_1	June 1, 1939	2.5	14.0
2	Diabetic with cardiac failure	Nov. 17, 1938	1.0	6.0	" " "	Dec. 7, 1938	5.5	17.0
3	Reactive depression	June 5, 1939	2.5	3.5	" " "	June 12, 1939	5.0	7.0
4	Polyneuritis; adrenal disease	Nov. 2, 1938	1.5	6.5	Dietary	Nov. 28, 1938	8.5	12.0
5	Diabetic neuritis	May 25, 1939	2.5	3.0	Crystalline vitamin B_1	June 2, 1939	5.0	12.0
		" 31, 1939	1.0	2.5				
6	Alcoholism with Korsakoff's psychosis	June 22, 1939	0.0	2.5	" " "	" 28, 1939	2.0	5.0
7	"Alcoholic" polyneuritis and Wernicke's encephalopathy	July 24, 1939	0.0		" " "	Aug. 2, 1939	11.0	
8	Pellagra with peripheral neuritis	May 5, 1939		2.0	" " " + nicotinic acid	June 13, 1939	4.5	5.0
9	Pellagra with neurological changes	" 22, 1939	3.5	3.0	Nicotinic acid	May 24, 1939	1.0	2.0

We conclude from the above evidence that, except in occasional subjects who are probably unable adequately to phosphorylate the vitamin and also in those who have recently received intensive therapy with vitamin B_1 over a short period of time, the amount of cocarboxylase in the blood runs closely parallel to the total amount of vitamin B_1.

Variation of Amount of Cocarboxylase in Blood with Red and White Cell Count—We have previously stated (2) that "variations in the cell count, whether red or white, must be large before appreciably affecting the blood level of cocarboxylase. An increased

TABLE II

Variation of Values for Cocarboxylase with Blood Cell Counts

Diagnosis	Red blood cells	White blood cells	Cocarboxylase
	millions per microliter	*thousands per microliter*	*γ per 100 ml.*
Pernicious anemia	1.40	6.3	1.5
" "	1.43	2.6	4.5
Macrocytic " of pregnancy	1.98	7.5	3.0
Pernicious "	2.26	8.2	4.0
Idiopathic hypochromic anemia	2.30	4.2	4.0
Macrocytic anemia of pregnancy	2.98	7.1	3.0
" " " "	3.07	6.1	4.5
Pernicious "	3.63	4.1	4.0
Erythroblastosis with anemia	3.66		5.5
Ulcerative colitis	3.89	10.2	4.0
Pernicious anemia	3.89	4.8	2.0
" "	4.12	3.2	3.5
Neuritis (cured)	4.62	7.8	6.5
Osteomalacia	5.37	7.8	6.0

blood value can be readily observed with an increase in the polymorphonuclear leucocyte count of 20,000 or with an increase in the erythrocyte count of 2,000,000 per microliter." In Table II we have listed the diagnoses, the erythrocyte and leucocyte counts, and the cocarboxylase values obtained in fourteen patients. We have, of course, excluded from Table II all those subjects who presented clinical syndromes (other than anemia) that could be attributed directly to deficiency of any one of the water-soluble vitamins. Subjects with leucemia have also been excluded, since we are not here concerned with extreme variations in cell count.

All the remaining subjects, the records of whose blood counts we possess, are included. The correlation coefficient of the erythrocyte counts (which ranged from 1.4×10^6 to 5.4×10^6 per microliter) and the cocarboxylase values is 0.54 ± 0.20, $t = 2.7$. Although this is suggestive of a direct relationship between the two, the effect of the erythrocyte count upon the cocarboxylase value is not sufficiently great within this range of counts to necessitate correcting the value.

In this same group of patients, there was no correlation between the leucocyte counts, which ranged from 2600 to 10,200 per microliter, and the cocarboxylase values. The correlation coefficient was 0.20 ± 0.29. This also confirms our previous statement, quoted above.

We conclude that determinations of the amount of cocarboxylase in blood provide, within the limitations mentioned, a reasonably accurate index of the degree of saturation of the body with cocarboxylase and vitamin B_1. In the following pages our results in various clinical conditions have been assessed on this basis.

Use of Blood Cocarboxylase Value As a Measure of Saturation of Tissues with Vitamin B_1—Our results on 117 subjects are charted (Fig. 1) in groups according to the clinical diagnoses that had been made before the results of the determinations were known. We have previously found (2) that the average values for cocarboxylase in the blood of twenty-six young healthy adults ranged from 4.5 to 12.0 micrograms per 100 ml. with a mean ($\pm\ \sigma$) of 7.0 ± 2.1. Judged by this range, a value below 3.0 micrograms may be assumed to be definitely low; in Fig. 1 a line is drawn at this level, and a dotted line at the lowest normal value. Of the 111 untreated patients included in this series, 40 gave significantly low values, and 66 gave values below the lowest normal value.

The etiological relation of deficiency of vitamin B_1 to "alcoholic" polyneuritis has been demonstrated by several workers (6). This relation is supported by our results: values from four such patients are significantly low, and the other two give a figure (3.0 micrograms per 100 ml.) that is border line. One of us has obtained significantly low values for total vitamin B_1 in such cases (7, 8). In Fig. 1, the cocarboxylase values of two "uncomplicated" alcohol addicts are recorded; both are normal.

Cases of nutritional polyneuritis are also associated with low

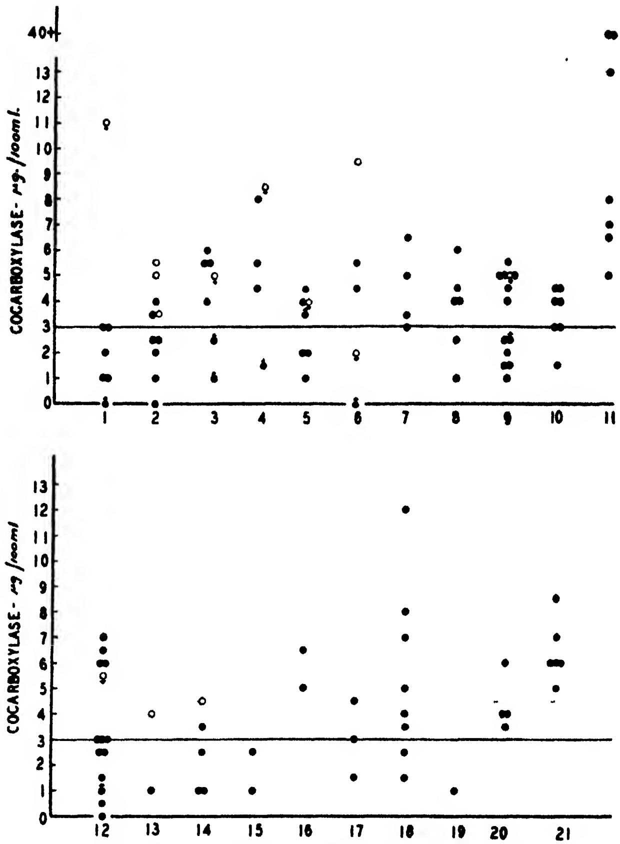

FIG. 1. Blood cocarboxylase (in micrograms per 100 ml.) as a measure of saturation of the tissues with vitamin B_1. ● represents results with untreated patients; ○ results with patients who have received vitamin B_1 parenterally. The solid line is drawn at the level considered definitely low; the dotted line at the lowest normal value. The column numbers (indicated by the figures on the horizontal scales) denote the following conditions: (1) alcoholic polyneuritis; (2) nutritional polyneuritis; (3) diabetic polyneuritis; (4) other cases of polyneuritis; (5) subacute combined degeneration of the cord; (6) Korsakoff psychosis; (7) acute confusional psychosis; (8) schizophrenia; (9) depression; (10) anemias; (11) leucemias; (12) cardiac failure or edema; (13) scurvy; (14) pellagra; (15) non-tropical sprue; (16) alcoholism (uncomplicated); (17) liver disease; (18) thyrotoxicosis; (19) myxedema; (20) ulcerative colitis; (21) miscellaneous diseases.

values. Only one of five cases of diabetic neuritis gave significantly low values (1.0 and 2.5 micrograms per 100 ml.). This subject, who was also an alcohol addict, was the only diabetic from whom we were able to obtain blood within a few weeks of the onset of an acute neuritis. Following administration of insulin and vitamin B_1, his neuritic signs and symptoms disappeared, and this was associated with a rise in the cocarboxylase value to 5.0 micrograms per 100 ml. Owing to the multiple therapy that he received, it is not possible to say that the neuritis was definitely caused by his undoubted deficiency of cocarboxylase. As nerve cells depend mainly upon carbohydrate as a source of energy, and as both cocarboxylase and insulin (and also the diabetogenic factor of the anterior pituitary) play a part in the utilization of carbohydrate, there are several possible ways in which the diabetic patient might develop neuritis. Further, diabetics that are treated with high carbohydrate diets need more vitamin B_1, and diuresis also increases the requirement. In some cases, arteriosclerosis probably also plays a part.

Among the other cases of polyneuritis is a subject who presented a syndrome resembling that described by Cushing, possibly of adrenal origin (Subject 4 in Table I). The return of the cocarboxylase value to normal coincided with the disappearance of his neuritis.

Results on various neurological and psychiatric conditions are plotted in Columns 5 to 9 (Fig. 1). Six untreated patients with pernicious anemia and subacute combined degeneration of the cord gave values ranging from 1.0 to 4.5 micrograms per 100 ml.; three are significantly low. We do not believe that these low values are necessarily of etiological importance in this disease, since cases of pernicious anemia without cord involvement have given similar results (1.5 to 4.5 micrograms per 100 ml.). Low erythrocyte counts may partly contribute to the low values, but the main factors are probably gastrointestinal dysfunction and poor dietary habits. No convincing evidence has ever been presented that vitamin B_1 plays a part in the etiology of subacute combined degeneration of the cord, although occasional clinicians have reported improvement after treatment with the vitamin. Nevertheless peripheral neuritis is part of the disease (9), and it may be that vitamin B_1 deficiency plays a part in the causation of this.

Of the samples from three patients with "untreated" Korsakoff psychosis, only one was sent with this diagnosis definitely established. This patient's blood contained 0.0 microgram per 100 ml. (total vitamin B_1 2.5 micrograms); after parenteral therapy with crystalline vitamin B_1 for 5 days, the cocarboxylase increased only to 2.0 micrograms, and the total vitamin B_1 was 5.0.

The values obtained on four cases of acute confusional psychosis, six of schizophrenia, and twelve of depression are plotted in Columns 7, 8, and 9 (Fig. 1). Many of these show significantly low values. However, it appears just as reasonable to suppose that the deficiency state developed after the onset of the mental disease as that it preceded it. That deficiency of vitamin B_1 may, in some instances, play a part in the etiology of mental depression is suggested by the following case report.

W., a 40 year-old, hypomanic, white male, a travelling salesman by profession, was admitted to the hospital with a reactive depression of acute onset. A blood sample drawn on the day of admission showed a cocarboxylase level of 2.5 micrograms per 100 ml. (total vitamin B_1 3.5 micrograms). Treatment was immediately started with parenteral administration of 10 mg. of crystalline vitamin B_1 daily. Improvement was rapid and the patient was discharged from the hospital recovered, 1 week after admission. The blood cocarboxylase level at this time was 5.0 micrograms per 100 ml. (total vitamin B_1 7.0).

Four subjects with lymphatic leucemia and one with erythroblastosis had cocarboxylase values within the normal range. Two subjects with myeloid leucemia had values well above the normal limits.

Six out of thirteen subjects with heart failure or edema had significantly low cocarboxylase values, and three gave border line values (3.0 micrograms). Two alcohol addicts with edema and one with acute cardiac failure had values of 3.0, 1.5, and 0.0 micrograms per 100 ml. respectively. One patient with a gastric ulcer and edema had a value of 0.5 microgram. One diabetic with congestive heart failure had a value of 1.0 microgram which increased to 5.5 micrograms after therapy with vitamin B_1. A case diagnosed as "wet beriberi" and one of "unexplained edema" had values of 3.0 and 2.5 micrograms respectively. A case of coronary thrombosis with gross edema gave a value of 3.0 micrograms and one of chronic glomerular nephritis, with anasarca,

gave values of 3.0 and 2.5 micrograms per 100 ml. (see Table I). There can be little doubt that, in most of these cases, the association of the deficiency of vitamin B_1 with the cardiovascular disturbances was more than coincidence. Of the four subjects who had normal blood cocarboxylase values, one had thyrotoxicosis with congestive heart failure; one organic heart disease (type not recorded) with congestive failure; one ulcerative colitis with nutritional edema; and the fourth was a 16 month-old female, with esophageal stenosis, edema, and anemia, who had been receiving parenteral liver therapy.

All of the seven untreated subjects with other deficiency diseases listed in Columns 13, 14, and 15 (Fig. 1) had low blood cocarboxylase values, showing clearly that deficiency diseases in man tend to be multiple. That the low cocarboxylase values in the cases of "non-tropical sprue" (idiopathic steatorrhea) were probably not due to an inability of the tissues to phosphorylate the vitamin is indicated by the fact that the values for total vitamin B_1 were also significantly low in these patients. The deficiency of vitamin B_1 in these patients must have been due to dietary deficiency, to increased destruction of the vitamin (in the gut or in the body), or to failure of absorption from the gut.

Although there is little doubt from the work of Alvarez (10) and others that deficiency of vitamin B_1 does not affect gastric acidity in man, achlorhydria is very commonly found in patients who are deficient in the vitamin. Laurent and Sinclair (11) described a subject with carcinoma of the stomach causing pyloric stenosis, who developed peripheral neuritis accompanied by a very low value for total vitamin B_1 in the blood, although he was taking the vitamin orally; after gastrojejunostomy and partial gastrectomy the polyneuritis disappeared and the vitamin in the blood returned to normal. These authors suggested that some factor produced by the stomach or duodenum might have destroyed or prevented the absorption of the vitamin in the prolonged presence of alkali. Later Sinclair was able to demonstrate destruction of the vitamin when incubated *in vitro* with gastric or duodenal juice from patients with achlorhydria, and Mahlo (12) showed that the presence of hemin causes destruction even in an acid medium. There is, therefore, evidence, both circumstantial and direct, that gastric achlorhydria tends to produce deficiency of vitamin B_1.

The results of gastric analyses were recorded in twenty of the patients in our present study; of these four had hypochlorhydria and twelve had achlorhydria, and of these sixteen patients with diminished gastric acidity thirteen had significantly low values for cocarboxylase in blood. The mean ($\pm\ \sigma$) of the sixteen values was 3.02 ± 1.37, whereas the mean of twenty-six normal values was 7.0 ± 2.1. The standard error of the difference between these two means is 3.98 ± 1.35 ($t = 2.9$). This result shows that in man there is a definite association between diminished gastric acidity and deficiency of cocarboxylase.

SUMMARY

1. Evidence has been presented to show that the amount of cocarboxylase in blood, determined by a chemical method, varies directly with the amount of total vitamin B_1, determined by a biological method, and also with the degree of saturation of the tissues with the vitamin. The only exceptions are the rare cases in which the ability of the tissues to phosphorylate the vitamin is apparently diminished, the cases in which the blood cell count is greatly increased (*e.g.*, cases of polycythemia vera or myeloid leucemia), and patients who have recently received intensive therapy with vitamin B_1 over a very short period of time. In most clinical cases, the determination of the amount of cocarboxylase in blood provides a rapid and reliable method of estimating the degree of saturation of the tissues with the vitamin.

2. There is an etiological relationship between deficiency of vitamin B_1 and "alcoholic" neuritis, nutritional neuritis, and some cardiovascular disturbances. The possible relation of deficiency of vitamin B_1 to diabetic neuritis is discussed.

3. Deficiency of vitamin B_1 is associated with cases of anemia, of subacute combined degeneration of the cord, and of certain psychiatric disorders, and the possible interpretations of the significance of this association are briefly discussed.

4. Diminished gastric acidity tends to be associated with deficiency of vitamin B_1.

5. Our investigations have shown that definite deficiency of vitamin B_1 is not uncommon among hospital patients in England; and we wish to emphasize that vitamin deficiencies in man are almost always multiple and are therefore usually inadequately treated by administration of a single pure vitamin.

We are very grateful to Professor R. A. Peters, for his interest and helpful advice throughout this work. For samples of blood from hospital patients we have been entirely dependent upon the generosity of clinicians at various hospitals. We wish to thank particularly Professors L. T. Witts, H. P. Himsworth, and H. Cairns, and Dr. C. C. Ungley, Dr. A. G. Cooke, Dr. A. H. T. Robb-Smith, and Dr. P. Berkenau. We are grateful to Mr. L. C. Waters for skilled assistance, and to the Christopher Welch Trustees for a grant to defray part of the cost of apparatus.

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RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM

IX. THE INFLUENCE OF AGE ON THE PHOSPHOLIPID METABOLISM OF VARIOUS PARTS OF THE CENTRAL NERVOUS SYSTEM OF THE RAT. THE COMPARATIVE PHOSPHOLIPID ACTIVITY OF VARIOUS PARTS OF THE CENTRAL NERVOUS SYSTEM OF THE RAT*

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The rate of deposition and loss of labeled phospholipid following the administration of sodium phosphate marked by the inclusion of radioactive phosphorus has been employed in this laboratory for comparing the phospholipid activity of various tissues (1-5). Brain shows a much slower deposition of radioactive phospholipid than liver, kidney, or small intestine (4). In the latter group of tissues the maximum amount of labeled phospholipid was found 10 to 15 hours after the ingestion of labeled phosphate, whereas in the brain a progressive increase in the content of radioactive phospholipid was observed for as long as 200 hours. Once the maximum amount of labeled phospholipid has been deposited, its loss from the brain occurs at a very slow rate. This slow but prolonged increase and retarded loss of labeled phospholipid characterize a low rate of phospholipid turnover.

It was also observed that the brains of younger rats incorporate phospholipid in greater amounts than those of older animals (4), and in the present study this variation in phospholipid activity with age has been investigated in detail in the following divisions of the central nervous system: *forebrain*, *cerebellum*, *medulla*,

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and *cord*. It was found that in all parts of the brain phospholipid activity varied with the age of the animal. It is highest at birth through the entire central nervous system. Although this general characteristic is shared by all the parts investigated, the phospholipid activity is by no means uniform throughout the system; striking differences are encountered in the activities of forebrain, cerebellum, medulla, and spinal cord.

EXPERIMENTAL

The data recorded here were obtained from over 250 animals. Rats of the following weights were investigated: 5 to 6 gm. (new born), 15 gm. (7 days old), 25 gm. (14 days old), 30 gm., 50 gm., 100 gm., 200 gm., and 300 gm. The weights of the animals within each group did not vary by more than 5 per cent except in the 5 and 300 gm. groups, in which the maximum differences in weight were about 10 per cent.

Radioactive phosphorus was administered subcutaneously as an isotonic solution of Na_2HPO_4; each rat received 1 cc. of this labeled phosphate solution per 100 gm. of body weight. The 5 gm. rats were injected between 2 and 4 hours after birth.

The adult rats were fed a standardized stock diet, and access to it was not interrupted during the course of the experiment. The young rats were kept with their mothers, and suckling was not interfered with after the administration of the labeled phosphorus.

The brains were removed 24 and 48 hours after the injection of the phosphorus. The phospholipid activities found, however, are referred to the age or weight of the rat at the time of injection.

Divisions of Brain and Treatment—The animals were sacrificed by decapitation and the brain removed immediately. The brain, including the olfactory lobes, was raised from the floor of the cranium and the nerves cut close to the base. The hypophysis was not included. Analyses were made of the following divisions: forebrain, cerebellum, medulla, and cord. These were obtained as follows:

Forebrain—The brain was divided at the line between the two pairs of corpora quadrigemina. The part superior to and including the upper pair of the corpora quadrigemina has been termed here the forebrain.

Medulla[1]—This included the lower pair of corpora quadrigemina and all tissue to the level of the first cervical nerve.

Cerebellum—This part was lifted directly off the medulla.

Spinal Cord—This included all tissues from the level of the first cervical nerve to the upper level of the cauda equina. The latter was not included.

The membranes (with the exception of the pia mater) and other extraneous material were removed from each part of the brain before it was immersed in alcohol.

Pools of these brain divisions were obtained from five to ten animals, most of the pools from six. In the case of 5, 15, and 25 gm. rats pools were always made from litter mates.

The method of extraction of phospholipid from these parts of the brain as well as the manner in which their radioactivity was determined has been recorded elsewhere (4).

All values shown in Figs. 1 to 5 represent averages of three or more separate analyses in which close agreement was obtained.

Phospholipid Activity[2] *at Various Ages*

Forebrain—The phospholipid activity of the forebrain found at 24 and 48 hours after the administration of radioactive phosphorus is shown in Fig. 1. The change in activity that accompanies growth of the animal is indeed striking. In the day-old[3] rat the average activity of the forebrain at the 24 hour interval was 0.53 per gm., but at the end of 1 week (15 gm. rat) the average activity of this tissue had fallen to 0.27 per gm., a value about 50 per cent lower. The most rapid decline in activity occurred between birth

[1] Separation of the above parts was effected with ease in all age groups studied except in the new born (5 to 6 gm.) rat. In this case difficulty was experienced in making a distinct separation between cerebellum and medulla. It was therefore deemed advisable to combine these two parts of the brain in the new born rat.

[2] The term phospholipid activity refers to the percentage of administered labeled phosphorus found in the form of phospholipid. Throughout this study activity per gm. is recorded.

[3] The average weight of an animal referred to throughout this study represents the age or weight *at the time radioactive phosphorus was injected.* But the animals were killed and the brain removed for analyses at intervals of 24 and 48 hours thereafter. New born rats gained about 1 gm. in 24 hours and 2 gm. in 48 hours.

and the time the rats attained a weight of 50 gm. The forebrain of the 50 gm. rat has lost over 90 per cent of the activity or gm. of tissue found at birth. By the time the rat attains a weight of 300 gm., the phospholipid activity of this part of the brain has fallen to a value approximately 2 per cent of that observed at birth.

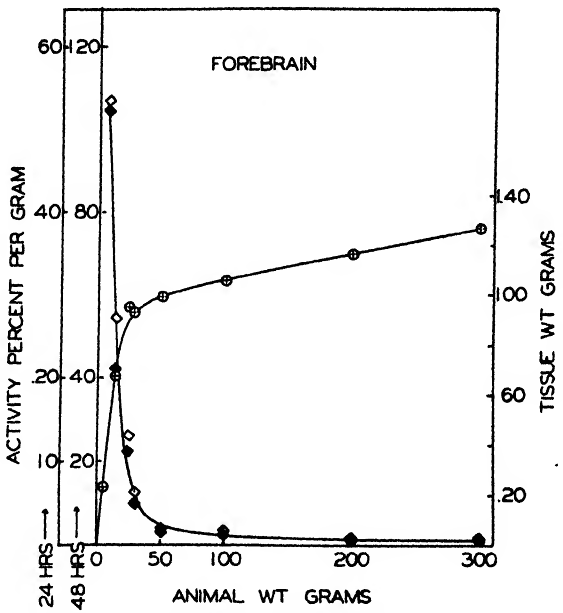

Fig. 1. The phospholipid activity of the forebrain. The ordinates on the left refer to per cent of labeled phosphorus found as phospholipid per gm. of forebrain at intervals of 24 and 48 hours after the administration of the radioactive phosphorus. ◇ represents average values obtained for phospholipid activity at the 24 hour interval; ◆ at the 48 hour interval. All values recorded here represent averages of three or more separate analyses. ' The growth of the forebrain from birth till the time the rat attains a weight of 300 gm. is represented by the symbol ⊕ and the ordinates for these values are shown on the right.

A similar course of events was found when the forebrain was examined at the 48 hour interval after the administration of radioactive phosphorus. The phospholipid activities at this later interval, however, were 1.5 to 2 times as great as those observed at 24 hours.

Spinal Cord—24 hours after the administration of radioactive phosphorus the average phospholipid activity of the cord of the new born rat was 1.11 per gm. (Fig. 2). By the end of the 1st week (15 gm. rats) the average 24 hour activity had dropped to

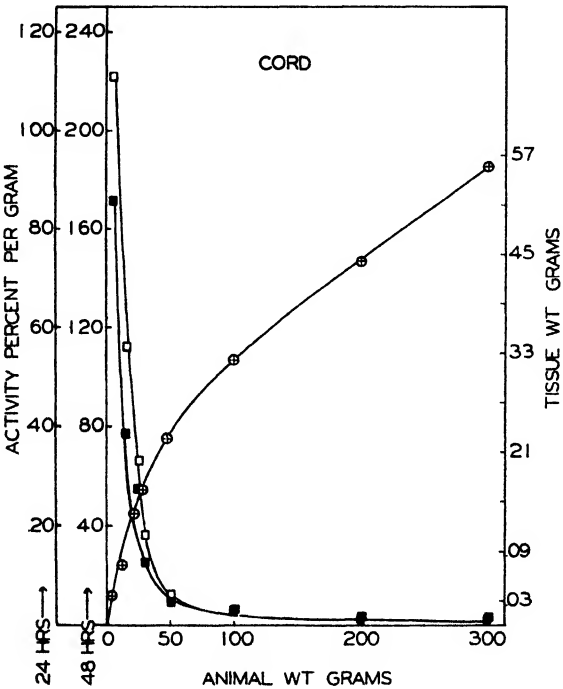

FIG. 2. The phospholipid activity of the spinal cord. The ordinates on the left refer to per cent of labeled phosphorus found as phospholipid per gm. of spinal cord at intervals of 24 and 48 hours after the administration of the radioactive phosphorus. □ represents average values obtained for phospholipid activity at the 24 hour interval; ■ at the 48 hour interval. All values recorded here represent averages of three or more separate analyses. The growth of the spinal cord from birth till the time the rat attains a weight of 300 gm. is represented by the symbol ⊕ and the ordinates for these values are shown on the right.

0.56 per gm. Thus in the short space of 7 days the cord lost about 50 per cent of the activity present at birth. At 2 weeks of age (25 gm. rats) the activity per gm. was 0.33, and this precipitous drop continued until the rat reached a weight of 50 gm.

From 50 gm. on the rate of loss in activity was less rapid than at earlier ages, although it should be noted that the activity found in the cord of the 100 gm. animal was approximately 50 per cent of that found in the 50 gm. rat. The activity observed in the cord of the 300 gm. rat was 0.01 per gm., a value that is less than 1 per cent of that found at birth.

The activities at the 48 hour interval after phosphorus administration were in all cases higher than at the 24 hour period. Here again the value found per gm. of tissue for the 300 gm. animal was less than 1 per cent of that observed in the new born rat.

Cerebellum—The value shown in the legend of Fig. 3 for the new born rat (5 gm.) was obtained from the analysis of cerebellum and medulla combined. As noted above, separation of these parts was not made in the 5 gm. rat. In all other age groups examined separate analyses of cerebellum and medulla were obtained. The rapid drop in activity found for cord and forebrain in rats of from 5 to 50 gm. was also observed in the case of the cerebellum. Thus in the 15 gm. rat 0.36 per cent of the administered labeled phosphorus was found as phospholipid in each gm. of cerebellum, whereas in the 50 gm. rat the cerebellum incorporated 0.035 per cent of the phosphorus into phospholipid per gm. of tissue.

Medulla—The changes in phospholipid activity found in the medulla (Fig. 4) of the growing rat paralleled those observed in the other brain divisions. The most striking loss in activity occurred during the interval between birth and the time the rat attained a weight of 50 gm. The medulla continued to lose activity as the animal grew beyond 50 gm., but the loss during this time was less striking than the earlier one.

Comparison of Rates of Loss of Phospholipid Activity That Occur with Age in Forebrain, Cerebellum, Medulla, and Spinal Cord—Although the phospholipid activities present at birth differ widely in these four divisions of the central nervous system, yet from birth until the time the rat reaches a weight of 50 gm. the rate at which the activities decrease is roughly similar (Figs. 1 to 4). At some point between 30 and 50 gm. an abrupt change in phospholipid activity occurs. The labeled phospholipid activities per gm. of forebrain, cerebellum, medulla, and spinal cord found in rats between the weights of 50 and 300 gm. are recorded in

Fig. 5. Activity continues to be lost throughout the central nervous system as the animal grows older, but the loss observed

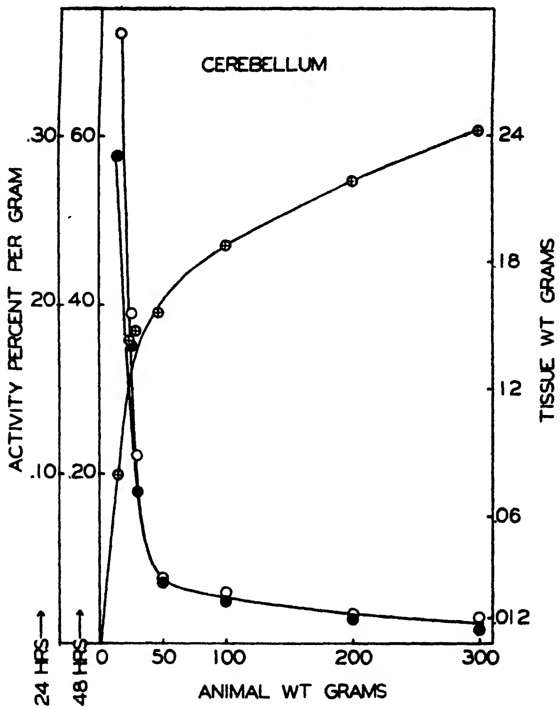

Fig. 3. The phospholipid activity of the cerebellum. The ordinates on the left refer to per cent of labeled phosphorus found as phospholipid per gm. of cerebellum at intervals of 24 and 48 hours after the administration of radioactive phosphorus. O represents the average values obtained for phospholipid activity at the 24 hour interval; ● at the 48 hour interval. As noted in the text, the cerebellum and medulla were combined for analyses in the 5 gm. rat and the average activities per gm. were 0.61 at the 24 hour interval and 1.08 at the 48 hour interval. All values recorded here represent averages of 3 or more separate analyses. The growth of the cerebellum from birth till the time the rat attains a weight of 300 gm. is represented by the symbol ⊕ and the ordinates for these values are shown on the right.

after the rat weighs 50 gm. is much more gradual than in younger rats.

Fig. 5 shows that the rate of drop in phospholipid activity after

the time the rat reaches a weight of 50 gm. is no longer uniform throughout the various divisions of the central nervous system,

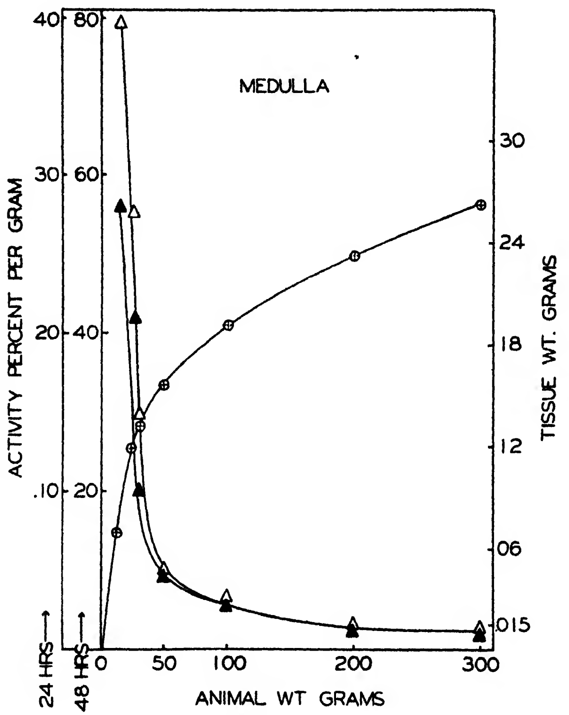

FIG. 4. The phospholipid activity of the medulla. The ordinates on the left refer to per cent of labeled phosphorus found as phospholipid per gm. of medulla at intervals of 24 and 48 hours after the administration of radioactive phosphorus. △ represents average values obtained for phospholipid activity at the 24 hour interval; ▲ at the 48 hour interval. As noted in the text, the medulla and cerebellum were combined for analyses in the new born rat (5 gm.) and the average activities per gm. were 0.61 at the 24 hour interval and 1.08 at the 48 hour interval. All values recorded here represent averages of three or more separate analyses. The growth of the medulla from birth till the time the rat attains a weight of 300 gm. is represented by the symbol ⊕ and the ordinates for these values are shown on the right.

the loss of activity in the cord being more precipitous than in the forebrain, cerebellum, or medulla.

Growth of Brain—The most striking observation presented here is the decline in phospholipid activity that occurs in the central nervous system between birth and the time the rat weighs 50 gm. This pronounced fall in activity is found during the interval in which the most rapid growth is occurring in the central nervous system. Thus in the 50 gm. rat the forebrain has attained 78

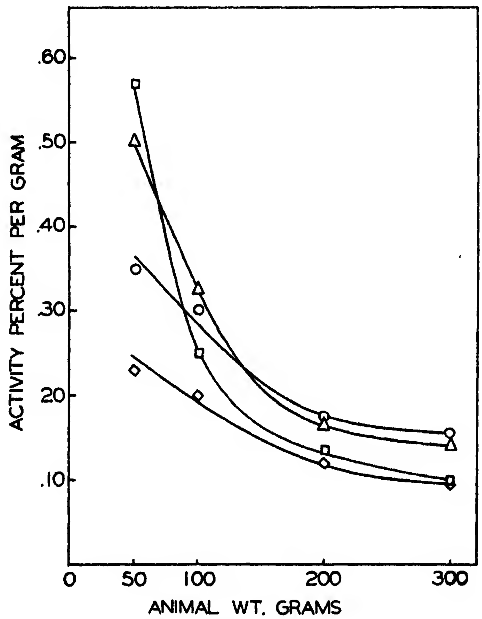

FIG. 5. Comparison of the decline in phospholipid activity of forebrain, cerebellum, medulla, and spinal cord between the times the rat reaches weights of 50 and 300 gm. The symbols have the same meaning as in Figs. 1 to 4 and represent activities 24 hours after the administration of labeled phosphorus. ◇ forebrain, ○ cerebellum, △ medulla, □ spinal cord.

per cent of the growth found in the 300 gm. rat (Fig. 1), the cerebellum 65 per cent (Fig. 3), the medulla 60 per cent (Fig. 4), and the cord 40 per cent (Fig. 2). The growth data shown in Figs. 1 to 4 were obtained from 70 pools involving over 400 rats, and the values recorded compare favorably with those found by Donaldson and Hatai (6), who, however, used brain divisions slightly different from those employed here. Those workers found

no sex difference in the growth of the various parts of the brain, while in the present study both sexes were used to provide the data of growth as well as of phospholipid activity.

Comparative Phospholipid Activity of Forebrain, Cerebellum, Medulla, and Cord

In order to compare the activities of the four divisions of the central nervous system the spinal cord has been taken as the

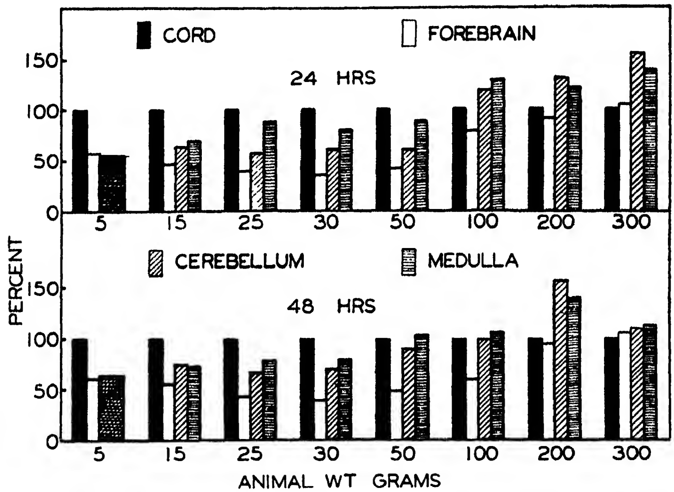

FIG. 6. The comparative phospholipid activities of forebrain, cerebellum, medulla, and spinal cord from birth till the time the rat attains a weight of 300 gm. In each age group, the spinal cord has been assigned the arbitrary value of 100. The cross-hatched areas for the 5 gm. rat represent the values for medulla and cerebellum combined.

standard and its activity in each weight group assigned an arbitrary value of 100 (Fig. 6). Although in the case of the 5 gm. rat 100 represents an *actual* phospholipid activity many times greater than 100 in the 50, 100, 200, etc., gm. rat, nevertheless this procedure permits a comparison of activities at any selected weight.

From birth until the time the rat attains a weight of 50 gm. (1 month) the cord is the most active part of the nervous system. During this time the cord is 2 or more times as active as the fore-

brain. The medulla approaches most closely the cord in activity.

In the older rats, namely those weighing 200 to 300 gm., a change in the relative activities of these divisions had occurred. 24 and 48 hours after the injection of radioactive phosphorus, the forebrain, cerebellum, and medulla were found to have higher activities than the cord.

The radioactive phosphorus used in this investigation was supplied by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due. The assistance furnished by the Works Progress Administration (Official Project No. 10482-A6) is also gratefully acknowledged.

SUMMARY

The deposition of labeled phospholipid at intervals of 24 and 48 hours after the administration of P^{32} was employed to compare the phospholipid activities of forebrain, cerebellum, medulla, and spinal cord.

1. The highest phospholipid activity is present on the day of birth in all parts of the central nervous system.

2. From birth until the time the rat attains a weight of 50 gm. a precipitous decline in phospholipid activity occurs through the entire central nervous system. So striking is this loss in phospholipid activity that by the time the rat reaches a weight of 50 gm. the spinal cord retains only 5 per cent of the activity present in the new born rat.

3. An abrupt change in phospholipid activity occurs in the central nervous system of the rat between the times it reaches weights of 30 and 50 gm. As growth proceeds beyond 50 gm., phospholipid activity decreases throughout the central nervous system, but at a much slower rate than was observed between birth and 50 gm. The spinal cord in the 200 gm. rat possesses an activity of 20 per cent of that present in the 50 gm. rat, whereas in the 300 gm. rat the cord retains 15 per cent of the activity found in the 50 gm. animal. The forebrain, cerebellum, and medulla also lose activity as the animal grows from 50 to 300 gm., but the rate of decline in activity is less than that which occurs in the cord.

4. Phospholipid activity is not uniform throughout the central

nervous system. It is highest in the spinal cord from birth to the time the rat reaches a weight of 50 gm. After this the *relative* activities of forebrain, cerebellum, and medulla rise steadily, and by the time a weight of 200 or 300 gm. is attained they are as great in those divisions as in the cord, or even greater.

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THE PICRIC ACID AND PICROLONIC ACID PRECIPITATES OF GONADOTROPIC EXTRACTS

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Fevold (1) has reported that the precipitates of the so called follicle-stimulating hormone with picrolonic, picric, and flavianic acids are inactive in promoting follicular growth and concludes that the acids are combined with the active substance in such a way as to render the prosthetic group inactive, the prosthetic group being again set free when the acid is removed. Similar results were obtained with the picrolonic acid precipitate of an unfractionated extract. Reactivated and nitrophenol-free material was obtained by dissolving the precipitate at pH 6.5 and precipitating with acetone.

It had previously been shown in this laboratory (2) that histone forms a precipitate with unfractionated pituitary extracts, the resulting mixture showing a decrease in activity similar to that found by Fevold for the picrate. In this instance, the histone forms an insoluble compound with inactive material, liberating the active material (pH 6.0 to 8.0). The decrease in activity observed was attributed to an increase in rate of resorption from the site of injection due to the precipitation of the naturally occurring adsorbing proteins, for the addition of zinc (3), which slowed resorption of the active fraction, produced the original physiologic effect. It occurred to us that the results obtained by Fevold with the picrolonic acid precipitate might be explained upon a similar basis, rather than upon a reversible chemical inactivation.

Extract and Precipitation—Freshly prepared aqueous extracts of a desiccated preparation of sheep hypophyses, corresponding to Powder A of Wallen-Lawrence (4) (proteins, insoluble at pH 8.5 and in 40 per cent ethanol, removed), were used. The precip-

itates were formed from aqueous solution of the hypophyseal preparation with aqueous saturated picric acid solution or acetone saturated picrolonic acid solution and were washed with

TABLE I

Influence of Divided Dosage and of Delayed Resorption on Physiologic Response to Picric Acid and Picrolonic Acid Precipitates of Gonadotropic Extract

The extract was administered for 4 days in each case.

Experiment No.	Extract administered	Total dose per rat	Times given daily	Average ovarian weight at end of experiment	No. of rats used to obtain ovarian weight average
		mg.		*mg.*	
I	Free aqueous extract	4	1	34	8
	Aqueous suspension of washed picric acid ppt. of extract	4	1	31	7
	Aqueous suspension of washed picrolonic acid ppt. of extract	4	1	23	8
II*	Control			14	4
	Free aqueous extract	1	1	19	4
	" " "	1	5	52	4
	Suspension of water-washed picric acid ppt. of extract	1	1	20	4
	Same	1	5	67	4
III*	Free aqueous extract + $CuSO_4$	1	1	122	5
	Suspension of water-washed picric acid ppt. of extract + $CuSO_4$	1	1	66	5
IV*	Free aqueous extract	1	5	67	6
	Suspension of water-washed picrolonic acid ppt. of extract	1	1	20	7
	Same	1	5	59	7
V*	Free aqueous extract + $CuSO_4$	1	1	126	8
	Suspension of water-washed picrolonic acid ppt. of extract + $CuSO_4$	1	1	106	8

* The preparations injected in these experiments were made isotonic with NaCl.

water as directed by Fevold (1). The solubility determinations were made with suspensions containing an equivalent of 1 mg. per cc. of original powder. The solutions or suspensions used for assay contained the dose per rat in 1 cc. concentration.

22 to 23 day-old rats were used for assay. A comparison of three to six groups of litter mates was made in each experiment to minimize variation in response. The extract was given once or five

TABLE II

Allocation of Active Material in Picric Acid and Picrolonic Acid Precipitation and Subsequent Elution

The extract was administered once daily for 4 days in each case.

Experiment No.	Extract administered	Total dose per rat in mg. equivalent to starting material	Cu per total dose added to test solution	Average ovarian weight at end of experiment	No. of rats used to obtain average ovarian weight
	Picric acid				
			mg.	*mg.*	
VI	Control	0		16	3
	1st wash water of picric acid ppt.	1	0.5	18	3
VII	Control	0		16	3
	Centrifuged saline extract of water-washed picric acid ppt., pH 4.0	0.8	0.5	38	3
VIII	Suspension of picric acid ppt.	1	0.5	64	4
	Filtrate of $CuSO_4$* extract of picric acid ppt.	1	0.5	66	4
	Suspension of ppt. of $CuSO_4$* extract of picric acid ppt.	1	0.5	23	4
	Picrolonic acid				
IX	Control	0		16	3
	1st wash water of picrolonic acid ppt.	1	0.5	17	6
X	Free aqueous extract	1	0.5	117	4
	Suspension of water-washed picrolonic acid ppt. of extract	1	0.5	106	8
	Centrifuged saline extract of water-washed picrolonic acid ppt., pH 4.5	1	0.5	65	8

* 0.05 per cent concentration of Cu.

times daily for 4 days as indicated in Table I, and in certain cases copper as copper sulfate was added to delay resorption and assure maximum response (3).

Results

The results of Experiment I, Table I, indicate that with the picrolonic acid precipitate less response is manifested than with the original aqueous extract, when administered once daily. In Experiment IV, however, in which greater response is obtained by dividing the dosage (twenty doses instead of four) there is no significant difference between the action of the original extract and the action of the picrolonic acid precipitate. When administered once daily, this amount (1 mg.) of the picrolonic acid precipitate produces a very slight effect. When the assay is made with copper in Experiment V, considerable activity is manifested by the picrolonic acid precipitate.

In Experiments I and II, Table I, there is no significant difference in the response to the free aqueous extract and in the response to the picric acid precipitate. Fevold gives no data for the effect of the picric acid precipitate of unfractionated extracts. Apparently there is no change in effect.

Experiments VI and IX, Table II, show that no detectable amount of active material is lost in the first wash water of the picric acid or picrolonic acid precipitates. Experiments VII and X show, however, that at about pH 4.5 the active material of the water-washed picric acid and picrolonic acid precipitates goes into solution in saline. The critical pH is apparently obtained by a second washing. Saline is not essential to effect solution (see Experiment VIII, Table II).

CONCLUSION

As a result of the foregoing experiments, it is concluded that picric acid and picrolonic acid completely precipitate the pituitary gonadotropic hormone and inactive material at about pH 3.0. At pH 4.5 the active material goes into solution, while some of the naturally occurring contaminating proteins are still insoluble. The picric acid and picrolonic acid precipitates retain their potency without removal of picric or picrolonic acid in the presence of copper, which presumably delays the resorption by adsorbing the active material at the pH of the body (3). Finally by administering the picric or picrolonic acid precipitates five times instead of once a day and producing a condition similar to delayed resorption, the full activity is demonstrated. This observa-

tion shows that the weak response to the picrolonic acid precipitate, when administered in the conventional assay, need not be explained on the basis of a reversible chemical inactivation.

SUMMARY

1. The active fraction of the picric acid and picrolonic acid precipitates of pituitary gonadotropic extract is insoluble at pH 3.0 and is soluble in isotonic saline at pH 4.5.

2. The insoluble picric acid and picrolonic acid precipitates or the soluble extracts (pH 4.5) retain their activity when assayed with Cu to delay resorption or when administered simply in divided dosage (five doses per day instead of one).

3. No evidence for a reversible chemical inactivation is thus obtained.

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THE B VITAMINS AND FAT METABOLISM

III. THE EFFECTS OF VITAMIN B_6 UPON LIVER AND BODY FAT

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Previous work has shown that rice polish concentrate, used as a dietary supplement in conjunction with thiamine and riboflavin, caused a marked increase in the body fat of rats (1). Bender and Supplee (2) found that, under such conditions, this concentrate had a supplemental effect upon body weight. They correlated the weight changes with the presence or absence of dermatitis, interpreting their results to mean that the factor in rice polish concentrate affecting growth was vitamin B_6. When crystalline vitamin B_6 became available, it was possible to determine whether this factor influenced weight and body fat in rats.

Methods

Young white rats of the Wistar strain, reared in the Connaught Laboratories colony, were employed. They were kept in individual screen bottom cages with water freely available. The animals were weighed daily. Groups of ten rats, five male and five female, were used and, unless otherwise stated, all results given are averages for such groups.

The rats were fed basal diets devoid of the vitamin B complex, low in choline, which is known to exert a marked influence upon liver fat, and low in casein because of the lipotropic action of this protein. The composition of the basal diets is given in Table I. After a 3 week depletion period the following supplements, dissolved in normal saline solution, were injected subcutaneously for a period of 1 week: thiamine hydrochloride (Merck) 12.5 micrograms, riboflavin (Hoffmann-La Roche) 10.0 micrograms, nicotinic acid (Eastman Kodak Company) 0.1 mg., vitamin B_6 (kindly donated by Merck and Company and by E. R. Squibb and Sons)

20 micrograms, choline (British Drug Houses) 10 mg. per rat per day. Extensive work in this laboratory has shown that all of these supplements have the same effect when injected as when given by mouth.

The animals were killed by stunning, the livers removed, and the total crude fatty acids in the bodies determined according to the method previously published (1). Owing to the much smaller amount of tissue in the liver, certain modifications of this method were made. The liver from each animal was placed in a tared 200 cc. round bottom flask with an extended neck, and the flask reweighed. To each were added 15 cc. of 60 per cent potassium hydroxide solution. The flasks were heated on a boiling water bath for 45 minutes. The flasks were removed from the bath

TABLE I

Composition of Basal Diets

Constituent	Diet 1	Diet 2
	per cent	*per cent*
Casein (Labco vitamin-free)	10	10
Sucrose	84	54
Triolein	0	30
Salt mixture (Steenbock-Nelson Salts 40 (3))	4	4
Agar	2	2
Cod liver oil concentrate	0.015	0.015

and 15 cc. of 95 per cent ethyl alcohol were added to each. After a reflux condenser was attached, the flask was reheated for 1 hour. The condenser was then removed and heating continued until the alcohol was evaporated. To each was added sufficient 40 per cent sulfuric acid solution to make the contents acid to litmus. The flasks were chilled and to each were added 25 cc. of petroleum ether and sufficient water to make the total volume 200 cc. From this point the procedure was the same as that used for the bodies.

EXPERIMENTAL

Series I—This experiment was designed to determine whether vitamin B_6 caused changes in body weight and in liver and body fat when supplied alone or in conjunction with various combinations of some of the known factors of the vitamin B complex and

with choline. The rats were fed a fat-free basal ration (Diet 1) *ad libitum* for 3 weeks, after which all groups, except one reserved as a control, received supplements and the same basal diet for 1

TABLE II

Effects of Vitamin B Supplements after Depletion Period

Series No.	Supplements	Average individual weight changes in 4th wk.	Total crude fatty acids	
			In body	In liver
		gm.	per cent	per cent
I. Fat-free diet	None, 3 wks. depletion	0	2.9	7.8
	" end of experiment	−10	1.9	3.6
	Vitamin B_6	−8	1.8	2.8
	Thiamine	+5	3.6	7.7
	" + vitamin B_6	+4	3.5	8.1
	" + choline	+3	3.8	4.0
	" + " + vitamin B_6	+3	3.4	3.0
	" + " + flavin	+8	5.3	3.8
	" + " + " + vitamin B_6	+13	6.1	4.1
	Choline + vitamin B_6	−8	2.0	2.5
II. Fat-free diet	None, 3 wks. depletion	0	1.7	3.2
	Nicotinic acid	−11	1.5	2.5
	Thiamine	+4	3.9	8.2
	" + nicotinic acid	+6	4.4	9.1
	" + choline	+6	4.2	3.1
	" + " + nicotinic acid	+5	4.3	2.7
	" + " + flavin	+5	4.3	3.6
	" + " + " + nicotinic acid	+8	4.5	3.0
	" + " + " + vitamin B_6	+12	4.8	3.3
	" + " + " + " " + nicotinic acid	+12	5.6	3.6
III. High fat diet	None	−5	3.2	6.0
	Thiamine	+7	7.3	15.0
	" + choline	+7	6.8	4.5
	" + vitamin B_6	+8	6.9	18.7
	" + " " + choline	+8	6.0	4.8

week. The average weight of the rats decreased from 96 to 71 gm. during the depletion period. The weight changes during the supplement period and the total crude fatty acids in the liver and body at the end of the experiment are given in Table II.

Series II—In this series the effect of nicotinic acid upon liver and body fat was investigated. It was employed because it is a constituent of the rice polish concentrate which we previously used. Nicotinic acid was given alone and with various combinations of other B vitamins and choline. The method used was the same as in Series I.

Series III—As a supplement to a diet free from fat and choline, thiamine will produce a greater than normal amount of fat in the liver (Series I). A high fat diet free from choline and supplemented with thiamine yields a still higher value for liver fat (4). In a study of vitamin B_6 deficiency in rats Halliday (5) found significantly heavier livers containing a higher percentage of total fatty acids in the deficient animals. The addition of choline remedied this condition to a large extent but even massive doses failed to bring the liver weight and total fatty acid content quite to normal. Series III was designed to show whether choline plus vitamin B_6 was more effective than choline alone in producing a normal amount of liver fat in rats receiving a high fat diet. The rats were depleted of their body stores of the vitamin B complex by feeding Diet 1 for 3 weeks. During the subsequent supplement period of 1 week Diet 2, containing 30 per cent triolein, was fed. Groups of twenty rats were used.

DISCUSSION

In Series I and II previous reports of the synthesis of fat from carbohydrate when thiamine was the only supplement were confirmed. Vitamin B_6 in conjunction with thiamine, choline, or the two together had no effect upon the amount of liver or body fat. When vitamin B_6 was combined with thiamine, choline, and riboflavin, the percentage of fat in the body was raised and the body weight was increased. Since we have been unable to determine the amount of vitamin B_6 present in the rice polish concentrate used in earlier work, we have been unable to compare the effects of the concentrate and of pure vitamin B_6. In Series II nicotinic acid had no consistent effect upon liver fat but had some effect in increasing body fat. Body weight was not altered by this substance.

In all three series, when choline was included as a supplement,

the percentage of fat in the liver was normal. This would indicate that choline was able to control the level of liver fat whether fat was supplied in the diet or whether it was formed by synthesis from carbohydrate when thiamine was furnished. The administration of vitamin B_6 with thiamine, in the absence of choline, has in no case resulted in a lowering of the high liver fat values ordinarily obtained with thiamine alone. In combination with choline, vitamin B_6 has apparently exerted no supplementary effect upon liver fat. These experiments offer no explanation for Halliday's finding that, in vitamin B_6-deficient rats, choline did not reduce the liver fat to normal.

In a recent paper Hastings, Muss, and Bessey (6) noted a decrease in liver metabolism associated with fatty infiltration when rats were kept on a basal diet without added yeast extract. When yeast extract was administered, neither effect was evident. The basal diet used by these workers would have been practically free of choline. The yeast extract, as prepared by them, would have contained a considerable amount of choline. The presence of choline would offer a possible explanation for the ability of yeast extract to prevent the development of fatty livers.

György and Goldblatt (7) have reported liver injury occurring in some rats kept on a diet deficient in the vitamin B complex and supplemented with thiamine, riboflavin, and a purified vitamin B_6 preparation. Here, again, the absence of choline in the basal diet and its presence in the two preventive substances, yeast and Peters' eluate fraction of yeast, may be significant.

SUMMARY

1. The administration of vitamin B_6 in conjunction with thiamine, riboflavin, and choline to rats fed a fat-free diet causes a slight increase in body fat and an increase in body weight.

2. Nicotinic acid slightly augments the effect of vitamin B_6 upon body fat but not upon body weight.

3. Neither vitamin B_6, nicotinic acid, nor riboflavin will prevent the deposition of fat in the liver which results when thiamine is administered. The amount of liver fat is normal if choline is administered, either alone or with any combination of the above factors.

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THERMODYNAMIC PROPERTIES OF SOLUTIONS OF AMINO ACIDS AND RELATED SUBSTANCES

IV. THE EFFECT OF INCREASING DIPOLAR DISTANCE ON THE ACTIVITIES OF ALIPHATIC AMINO ACIDS IN AQUEOUS SOLUTION AT TWENTY-FIVE DEGREES*

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This report, the fourth from an extended study of the thermodynamic properties of amino acids and related compounds, includes the osmotic and the activity coefficients, in aqueous solution at 25°, of β-alanine, *dl*-β-amino-*n*-butyric acid, γ-aminobutyric acid, *dl*-β-amino-*n*-valeric acid, *dl*-γ-amino-*n*-valeric acid, and of ε-aminocaproic acid.

Method and Materials

The method was the same as that employed in the determination of the activities of the amino acids previously reported (13, 14). The vapor pressures of the amino acid solutions were measured by the isopiestic technique described by Robinson and Sinclair (10). Sucrose was used as the reference standard.

The amino acids employed were recrystallized three to four times from water or water-alcohol mixtures. Their purity was checked by determination of the nitrogen content by a modified Kjeldahl method (7) or by repeated determinations of the solubility by a method employed in earlier studies.

Sucrose—The sucrose was a standard sample purchased from the Bureau of Standards.

β-Alanine—Purchased from the Department of Chemistry,

* Aided by a grant from the Fluid Research Fund of Yale University School of Medicine.

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University of Illinois. Solubility, 878.9 and 876.2 gm. per 1000 gm. of water.

dl-β-Amino-n-Butyric Acid—Purchased from the Department of Chemistry, University of Illinois. Nitrogen calculated, 13.6 per cent; found, 13.7.

γ-Aminobutyric Acid—Purchased from the Department of Chemistry, University of Illinois. Nitrogen calculated, 13.6 per cent; found, 13.0.

dl-β-Amino-n-Valeric Acid—Purchased from the Department of Chemistry, University of Illinois. Nitrogen calculated, 12.0 per cent; found, 11.8.

dl-γ-Amino-n-Valeric Acid—Purchased from the Department of Chemistry, University of Illinois. Nitrogen calculated, 12.0 per cent; found, 12.0.

ε-Aminocaproic Acid—Purchased from the Department of Chemistry, University of Illinois. Solubility, 890.5 and 894.7 gm. per 1000 gm. of water.

Sucrose Standard—In an earlier communication (13) it was stated that the osmotic coefficients for sucrose might require revision as more data appeared. Such a recalculation has now been made on the basis of the activity coefficients of potassium chloride as determined by Harned and Cook (6) and by Shedlovsky and MacInnes (12), using the isopiestic data on the ratio of sucrose to potassium chloride as determined by Robinson and Sinclair (10), Scatchard, Hamer, and Wood (11), and from certain measurements made in this laboratory. These three groups of determinations are in excellent agreement; hence the data from this laboratory are not given in detail. The osmotic coefficients of potassium chloride were estimated from the activity coefficients by graphical integration, and from these values and the isopiestic ratios the osmotic coefficients of sucrose were calculated. These values, represented by the equation $\varphi = 1 + 0.084m + 0.0104m^2 - 0.00237m^3 + 0.000115m^4$, are used in interpreting the data contained in this paper.

Results

The isopiestic molalities of the sucrose and amino acids are given in Table I. Large scale plots were made of the isopiestic ratios of the sucrose and amino acids, m_S/m_A, against m_S and

TABLE I

Concentrations, in Moles per 1000 Gm. of Water, of Isopiestic Solutions of Sucrose and Amino Acids at 25°

	m_S	m_A	m_S	m_A	m_S	m_A
β-Alanine	0.2153	0.2202	1.022	1.117	3.867	4.669
	0.2631	0.2691	1.095	1.205	4.075	4.912
	0.2953	0.3032	1.176	1.305	4.191	5.060
	0.3351	0.3464	1.264	1.402	4 386	5.299
	0.3414	0.3532	1.427	1.600	4.537	5.481
	0.3696	0.3823	1.614	1.825	4.842	5.862
	0.4075	0.4239	1.718	1.965	5.082	6.155
	0.4459	0.4659	2.194	2.549	5.280	6.389
	0.4767	0.4954	2.518	2.940	5 398	6.536
	0.506	0.530	2.621	3.094	5.502	6.664
	0.518	0.546	2.641	3.095	5.767	6.974
	0.562	0.593	2.670	3.164	5.774	7.009
	0.596	0.630	2.902	3.455	5.860	7 085
	0.607	0.642	3 145	3.756	6.177	7 465
	0.617	0.655	3.354	4.013	6.245	7.544
dl-β-Amino-*n*-butyric acid	0.2482	0.2530	1 247	1 329	3.270	3.678
	0.2639	0.2707	1.337	1 437	3 374	3.804
	0.2760	0.2817	1.451	1.567	3.433	3.895
	0.2933	0.3011	1.728	1.880	3 573	4 029
	0.3037	0.3107	1.768	1.929	3.784	4.295
	0.3376	0.3476	1.841	2.014	3.844	4.362
	0.3715	0.3833	1.895	2.077	3 947	4 480
	0.3940	0.4064	1.952	2.142	4.066	4.624
	0.4219	0.4372	2.057	2.266	4.144	4 711
	0.4401	0.4552	2.109	2.329	4.323	4.927
	0 4929	0 5092	2.158	2 385	4.468	5 101
	0.579	0.602	2.263	2 507	4 581	5 232
	0.631	0 657	2.446	2 722	4.722	5.402
	0.727	0.761	2.536	2.833	4.895	5.598
	0.737	0.770	2.537	2.829	5 018	5 760
	0.779	0.818	2.765	3.102	5.210	5.973
	0.829	0 869	2 880	3.239	5 850	6 739
	0.917	0.966	2.948	3 317	6.094	7.018
	1.033	1.094	3.015	3.397	6.427	7.409
	1.123	1.192	3.111	3.513	6 830	7.874
dl-β-Amino-*n*-valeric acid	0.2122	0.2141	1.247	1.298	2 969	3.247
	0.2503	0.2527	1.304	1.369	3.111	3.412
	0.3037	0.3076	1.402	1.469	3 333	3.673
	0.3840	0.3890	1.589	1.673	3.432	3.784
	0.4426	0.4477	1.745	1.862	3.546	3.924

TABLE I—*Continued*

	m_S	m_A	m_S	m_A	m_S	m_A
dl-β-Amino-*n*-valeric acid—*concluded*	0.4489	0.4563	1.841	1.967	3.715	4.119
	0.531	0.543	1.977	2.116	3.947	4.385
	0.535	0.545	2.005	2.145	4.328	4.841
	0.626	0.637	2.109	2.263	4.581	5.150
	0.779	0.798	2.263	2.443	4.744	5.328
	0.876	0.910	2.490	2.701	5.210	5.942
	0.960	1.000	2.536	2.751	5.850	6.749
	1.034	1 068	2.727	2.968	6.094	7.044
	1.103	1.150	2.886	3.140	6.427	7.477
γ-Amino-*n*-butyric acid	0.2523	0.2632	0.916	1.003	3.270	3.731
	0.2549	0.2635	1.033	1.135	3.348	3.814
	0.2884	0.3017	1.123	1.241	3.435	3.940
	0.2927	0.3033	1.247	1.387	3.512	4.014
	0.3481	0.3653	1.365	1.528	3.663	4.191
	0.3485	0.3641	1.400	1.566	3.715	4.248
	0.3496	0.3686	1.589	1.788	3.801	4.343
	0.3924	0.4120	1.728	1.947	3.947	4.530
	0.4041	0.4269	1.872	2.122	4.144	4.739
	0.4261	0.4500	1.909	2.161	4.217	4.829
	0.4489	0.4772	2.057	2.340	4.328	4.961
	0 4998	0.530	2.265	2.583	4.468	5.117
	0.508	0.543	2.446	2.786	4.581	5.246
	0.526	0.561	2.765	3.156	4.744	5.430
	0.637	0.684	2.853	3.259	5.018	5.752
	0.771	0.838	2.969	3.379	5.210	5.973
	0.784	0.849	3.069	3.514	5.652	6.469
	0.810	0.881	3.111	3 557	5.850	6.703
dl-γ-Amino-*n*-valeric acid	0.2126	0.2173	1.451	1.565	3.374	3.711
	0.2308	0.2359	1.745	1.896	3.435	3.790
	0.3202	0.3288	1.802	1.954	3.597	3.962
	0.3924	0.4072	1 977	2.149	3.663	4.037
	0.4542	0.4717	2.005	2.181	3.745	4.134
	0.4771	0.4965	2.158	2.343	3.820	4.220
	0.517	0.540	2.260	2.472	3.944	4.367
	0.576	0.603	2.343	2.564	4.025	4.438
	0.646	0.680	2.446	2.674	4.217	4.645
	0.685	0.719	2.556	2.807	4.328	4.794
	0.745	0.783	2.765	3.039	4.468	4.926
	0.800	0.848	2.772	3.044	4.648	5.159
	0.876	0.926	2.853	3.137	4.744	5.231
	0.931	0.988	2.866	3.144	4.895	5.410
	1.054	1.126	3.096	3.412	5.019	5.547
	1.103	1.180	3.111	3.428	5.210	5.755

TABLE I—*Concluded*

	m_S	m_A	m_S	m_A	m_S	m_A
ε-Aminocaproic acid	0.2152	0.2236	1.433	1.565	3.661	3.987
	0.2401	0.2512	1.502	1.642	3.867	4.221
	0.3178	0.3338	1.614	1.764	4.075	4.446
	0.3764	0.3990	1.783	1.950	4.212	4.596
	0.4075	0.4329	2.035	2.229	4.318	4.706
	0.4755	0.505	2.195	2.399	4.537	4.946
	0.534	0.572	2.446	2.678	4.842	5.284
	0.583	0.628	2.510	2.739	4.850	5.277
	0.631	0.681	2.621	2.863	5.082	5.538
	0.662	0.714	2.641	2.879	5.155	5.612
	0.821	0.890	2.902	3.170	5.398	5.892
	0.896	0.975	3.087	3.369	5.657	6.168
	0.966	1.051	3.145	3.437	5.992	6.543
	1.264	1.377	3.381	3.686	6.177	6.735
	1.344	1.470	3.539	3.868	6.245	6.814

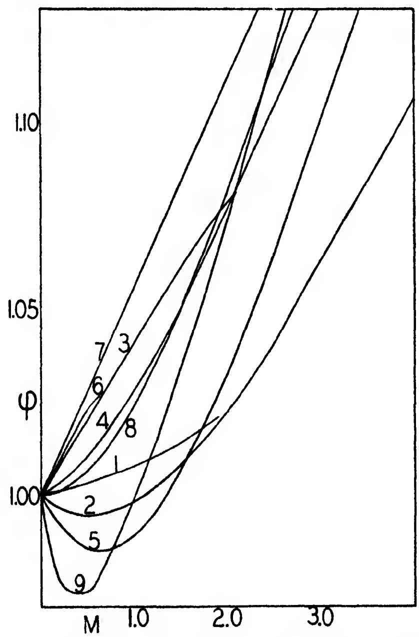

FIG. 1. Curve 1, *dl*-α-alanine, Curve 2, β-alanine, Curve 3, *dl*-α-amino-*n*-butyric acid, Curve 4, *dl*-β-aminobutyric acid, Curve 5, γ-aminobutyric acid, Curve 6, *dl*-α-amino-*n*-valeric acid, Curve 7, *dl*-β-amino-*n*-valeric acid, Curve 8, *dl*-γ-amino-*n*-valeric acid, Curve 9, ε-aminocaproic acid.

smooth curves were drawn through the points. The results were usually consistent within ±0.003 in the ratio, m_S/m_A.

Since the activity of water in the sucrose and in the amino acid solutions is the same at equilibrium, the osmotic coefficients of the amino acid solutions, φ_A, may be calculated from the relation $\varphi_A = \varphi_S(m_S/m_A)$.

TABLE II

Activity Coefficients

m	β-Alanine	*dl*-β-Amino-butyric acid	*dl*-β-Amino-valeric acid	γ-Amino-butyric acid	*dl*-γ-Amino-valeric acid	ε-Amino-caproic acid
0.2	0.994	1.006	1.016	0.983	1.001	0.971
0.5	0.988	1.019	1.044	0.966	1.009	0.951
0.7	0.987	1.029	1.064	0.961	1.018	0.946
1.0	0.988	1.049	1.095	0.960	1.037	0.942
1.2	0.991	1.064	1.118	0.964		0.964
1.5	1.000	1.089	1.154	0.975	1.079	1.002
2.0	1.016	1.138	1.218	1.006	1.133	1.072
2.5	1.042	1.193	1.289	1.050	1.199	1.140
3.0	1.073	1.254	1.364	1.104	1.273	1.208
3.5	1.109	1.319	1.443	1.165	1 355	1.271
4.0	1.149	1 388	1.523	1.230	1.440	1.330
5.0	1.239	1.525	1.681	1.374	1.622	1.436
6.0	1.339	1.662	1.818	1.506	1.812	1.517
6.5			1.870	1.575		1.556
7.0	1.445	1.790			2.014	

The results are shown in Fig. 1. The osmotic coefficients, except those of ε-aminocaproic acid, are represented, usually within ±0.001, by the following empirical equations.

β-Alanine—$\varphi_A = 1 - 0.018985m + 0.019307m^2 - 0.002431m^3 + 0.000109m^4$

dl-β-Amino-n-Butyric acid—$\varphi_A = 1 + 0.008244m + 0.02395m^2 - 0.00503m^3 + 0.0003335m^4$

dl-β-Amino-n-Valeric Acid—$\varphi_A = 1 + 0.0579m - 0.002374m^2 + 0.000775m^3 - 0.000102m^4$

γ-Aminobutyric Acid—$\varphi_A = 1 - 0.0402m + 0.04616m^2 - 0.00725m^3 + 0.000375m^4$

dl-γ-Amino-n-Valeric Acid—$\varphi_A = 1 - 0.000675m + 0.02885m^2 - 0.0047m^3 + 0.000246m^4$

By use of the empirical equations for the osmotic coefficients, $\varphi_A = 1 + am + bm^2 + cm^3 + dm^4$, where a may be positive or

negative, and b, c, and d may be positive, negative, or 0, the activity coefficients were calculated from the equation

$$2.3026 \log \gamma = 2\,am + 3/2\,bm^2 + 4/3\,cm^3 + 5/4\,cm^4$$

Since the osmotic coefficients of ϵ-aminocaproic acid were not represented by an equation, the activity coefficients of this amino acid were obtained by graphical integration. Table II is a summary of these data.

DISCUSSION

In the earlier studies of this series (13, 14) it was shown that the osmotic coefficients of the α-amino acids usually increased with each additional methylene group. This was probably not due to a change in electrical properties, since the dielectric constants of aqueous solutions of α-amino acids are nearly the same, increasing linearly with the concentration per liter of solution (15). However, inspection of Fig. 1 shows that there is considerable difference in the osmotic coefficients of amino acids of the same empirical formula. Such differences are probably related to differences in dipole distance; *i. e.*, the distance between the two charged groups of the amino acid in the zwitter ion form. In general the larger the dipole moment the lower the osmotic coefficient. This is opposite in effect to increasing the length of the carbon chain.

Cohn, McMeekin, Ferry, and Blanchard have recently related the activity coefficients of certain amino acids to the change in dielectric constant of their solutions (3). Their plot of $-\log \gamma/C$ against the ratio of the dielectric constant of the solvent to that of the dielectric constants of the solutions, D_0/D, shows a straight line relationship despite the widely varying activities of the substances studied. Similar relationships have been calculated for the amino acids reported here and the results are shown in Fig. 2. The values for the α-amino acids are plotted for comparison.

From the values of $-\log \gamma/C$ at $D_0/D = 1$ the relationships shown in Table III appear. The values for this function decrease with an increase in length of the carbon chain and increase with an increase in dipole moment. Cohn, McMeekin, Edsall, and Weare (2) and McMeekin, Cohn, and Weare (9) have studied the activities of some of these amino acids by comparing the solubility in water to that in certain organic solvents. Some of these results,

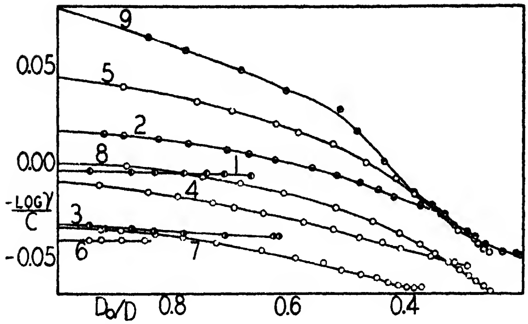

FIG. 2. Curve 1, *dl*-α-alanine, Curve 2, β-alanine, Curve 3, *dl*-α-amino-*n*-butyric acid, Curve 4, *dl*-β-aminobutyric acid, Curve 5, γ-aminobutyric acid, Curve 6, *dl*-α-amino-*n*-valeric acid, Curve 7, *dl*-β-amino-*n*-valeric acid, Curve 8, *dl*-γ-amino-*n*-valeric acid, Curve 9, ϵ-aminocaproic acid. The values of D_0 and those of D for Curves 1 to 4, 6, 8, and 9 are from Wyman and McMeekin (16), while those for Curve 5 are from Devoto (5) and those of Curve 7 are assumed to be the same as those for Curve 4. The partial molal volumes used in calculating C from m for Curves 1 to 4 and 9 are from Daniel and Cohn (4), those for Curve 8 from Cohn *et al.* (1), and those for Curves 5 to 7 were estimated to be 72.1, 92.7, and 92.7 respectively.

TABLE III

Functions Derived from Change of Activity Coefficient with Concentration and Solvent

NCH_2	$\frac{-\text{Log}\,\gamma}{C}$ at $\frac{D_0}{D} = 1$				$-\text{Log}\,\frac{N_A}{N_0}$†			
	α	β	γ	ϵ	α	β	γ	ϵ
0	0.096				3.391			
1	−0.005	0.015			2.856	3.139		
2	−0.033	−0.011	0.043		2.375			
3	−0.041	−0.035	−0.001					
4				0.079	1.414			2.972
	K_R^*				K_s^*			
0	0.092				−0.004			
1	0.010	0.022			0.015	0.007		
2	0.016	0.024	0.046		0.049	0.035	0.003	
3	0.000	0.012	0.018		0.042	0.047	0.019	
4				0.098				0.019

† These values are from solubility measurements in alcohol and water at 25° (2, 9).

expressed as the negative logarithm of the ratio of the solubility in mole fractions in alcohol to that in water, $-\log N_A/N_0$, are given in Table III. It is evident that $\log N_A/N_0$ varies with the length of the carbon chain and with dipolar distance in the same way that $\log \gamma/C$ does.

The values of $-\log \gamma/C$ for the α-amino acids varied linearly with D_0/D (3) and it was possible to estimate approximate interaction constants from the equation $-\log \gamma/C = K_R^*(D_0/D) - K_s^*$, in which the salting-in constant, K_R^*, is the slope of the line and K_s^*, the salting-out constant, is the intercept.[1] In the application of these equations to the present data, since the curves are not straight lines, it was necessary to use the slopes obtained from the measurements in dilute solutions. The values for the constants thus obtained are given in Table III. The values for salting-in constants increase with increase in dipole moment and in general decrease with increase in length of carbon chain, while those for the salting-out constant usually decrease with increase in dipole moment and increase with length of carbon chain.

The possible reasons for the curvature of the lines in Fig. 2 are several. Whereas straight lines were obtained for amino acids previously studied (15), it was recognized that the relationship was semiempirical and did not agree with the theoretical treatment (3). The measurements extend to much lower values of D_0/D than did the earlier measurements (3). The calculation is based on the assumption that dielectric constant increment is linear even at high concentrations (15), although actual experimental measurements at even moderate concentrations are lacking for most of these compounds (15, 16). In some cases it is assumed that the apparent molal volumes used in the estimation of C from m are the same at high concentrations as at the low concentrations at which they were measured.

The authors are indebted to Professor Edwin J. Cohn, of the Harvard Medical School, for many helpful suggestions in interpret-

[1] The values for $-\log f/C$ are smaller than those for $-\log \gamma/C$ by an appreciable but almost constant amount (approximately 0.009 up to 5 M for the substances studied in the present investigation) and may be calculated from the relation derived from that given by MacInnes (8), $f = \gamma(1 + 0.001mM_s)$, where f is the activity coefficient on a mole fraction basis, m = molality of solute, and M_s = molecular weight of solvent.

ing their results and to Professor David I. Hitchcock for valuable criticisms.

SUMMARY

Isopiestic vapor pressure measurements have been made at 25° of aqueous solutions of the following amino acids: β-alanine, *dl*-β-aminobutyric acid, *dl*-β-aminovaleric acid, γ-aminobutyric acid, *dl*-γ-amino-*n*-valeric acid, and ε-aminocaproic acid.

The osmotic coefficients and activity coefficients of these amino acids have been calculated.

Certain relationships between the activity coefficients and the dielectric constants of their solutions have been discussed.

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THERMODYNAMIC PROPERTIES OF SOLUTIONS OF AMINO ACIDS AND RELATED SUBSTANCES

V. THE ACTIVITIES OF SOME HYDROXY- AND N-METHYLAMINO ACIDS AND PROLINE IN AQUEOUS SOLUTION AT TWENTY-FIVE DEGREES*

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This report is of the determination by an isopiestic method of the osmotic and activity coefficients of *dl*-proline, *l*-hydroxyproline, *dl*-serine, *dl*-threonine, sarcosine, and of betaine in aqueous solution at 25°.

Method and Materials

The method was the same as that employed in previous studies (11–13), and consisted in measuring the vapor pressures of the amino acid solutions by the isopiestic technique of Robinson and Sinclair (10) with sucrose as the reference standard. With the exception of betaine the amino acids were recrystallized three to four times from water or water-alcohol mixtures. The betaine was prepared from betaine chloride and recrystallized from anhydrous methyl alcohol and ethyl ether. Their purity was checked either by determination of the nitrogen content or by determinations of the solubility in successive portions of the solvent.

Sucrose—The sucrose was a standard sample purchased from the Bureau of Standards.

dl-Proline—Purchased from Hoffmann-La Roche, Inc. Nitrogen calculated, 12.2 per cent; found, 12.6.

* Aided by a grant from the Fluid Research Fund of Yale University School of Medicine.

† Honorary Fellow in Physiology, 1933–39.

TABLE I

Concentration, in Moles per 1000 Gm. of Water, of Isopiestic Solutions of Sucrose and Amino Acids

	m_S	m_A	m_S	m_A	m_S	m_A
dl-Serine	0.1991	0.2065	0.2834	0.2987	0.4016	0.4335
	0.2047	0.2125	0.2925	0.3093	0.4047	0.4385
	0.2099	0.2176	0.3133	0.3332	0.4148	0.4501
	0.2164	0.2250	0.3505	0.3753	0.4212	0.4567
	0.2207	0.2299	0.3566	0.3810	0.4340	0.4734
	0.2257	0.2353	0.3614	0.3893	0.4409	0.4804
	0.2514	0.2628	0.3720	0.4010	0.4483	0.4894
	0.2720	0.2863	0.3922	0.4237	0.4538	0.4958
dl-Threonine	0.2546	0.2613	0.572	0.610	1.034	1.152
	0.2549	0.2616	0.615	0.656	1.190	1.348
	0.2944	0.3038	0.682	0.739	1.260	1.435
	0.3203	0.3313	0.732	0.796	1.365	1.569
	0.3481	0.3626	0.779	0.846	1.400	1.618
	0.4294	0.4512	0.810	0.882	1.641	1.943
	0.4311	0.4508	0.894	0.986	1.710	2.035
	0.543	0.577	0.918	1.011	1.769	2.114
l-Hydroxyproline	0.2207	0.2243	0.4991	0.521	1.337	1.489
	0.2546	0.2584	0.521	0.546	1.400	1.569
	0.2920	0.2986	0.563	0.590	1.402	1.578
	0.3050	0.3132	0.637	0.672	1.589	1.808
	0.3217	0.3303	0.768	0.820	1.710	1.955
	0.3481	0 3573	0.810	0.864	1.768	2.024
	0.3736	0.3841	0.943	1.027	1.790	2.052
	0.4051	0.4193	1.123	1.233	1.985	2.298
	0.4867	0.506	1.260	1.402	2.371	2.806
dl-Proline	0.2207	0.2338	1.247	1.312	3.069	3.380
	0.2494	0.2527	1.345	1.420	3.111	3.440
	0.2503	0.2540	1.400	1.484	3.268	3.630
	0.3217	0.3261	1.589	1.685	3.374	3.732
	0.3627	0.3677	1.769	1.891	3.422	3.804
	0.3715	0.3793	1.841	1.984	3.546	3.949
	0.4826	0.4919	1.866	1.998	3.573	3.960
	0.4867	0.4948	1.952	2.112	3.820	4.266
	0.543	0.554	2.007	2.160	3.947	4.389
	0.596	0.610	2.109	2.287	4.025	4.473
	0.622	0.634	2.263	2.471	4.328	4.835
	0.650	0.666	2.343	2.562	4.331	4.833
	0.734	0.753	2.446	2.674	4.468	4.997
	0.808	0.833	2.536	2.780	4.648	5.196
	0.917	0.948	2.651	2.915	4.744	5.298
	1.023	1.063	2.765	3.043	4.922	5.510
	1.086	1.134	2.827	3.097	5.429	6.077
	1.123	1.177	2.948	3.254	6.432	7.248

TABLE I—*Concluded*

	m_S	m_A	m_S	m_A	m_S	m_A
Sarcosine	0.2482	0.2528	1.433	1.572	3.512	4.082
	0.3367	0.3449	1.502	1.649	3.661	4.276
	0.3888	0.3994	1.783	1.986	3.777	4.422
	0.4205	0.4341	1.900	2.114	4.212	4.951
	0.4214	0.4364	1.909	2.136	4.318	5.084
	0.518	0.539	2.035	2.289	4.386	5.158
	0.552	0.574	2.179	2.458	4.842	5.723
	0.609	0.635	2.265	2.567	4.850	5.726
	0.678	0.710	2.431	2.769	5 155	6.093
	0.703	0.741	2.579	2.949	5.280	6.240
	0.772	0.816	2.630	3.014	5.398	6.385
	0.896	0.956	2.727	3.119	5.502	6.517
	1.043	1.116	2.901	3.344	5.767	6.810
	1.100	1.182	3.069	3.563	5.992	7.055
	1.244	1.346	3.254	3.776	6.177	7.296
	1.306	1.415	3.381	3.935	6.245	7.544
Betaine	0.2584	0.2515	1.502	1.391	3.254	2.920
	0 3449	0.3352	1.783	1.646	3.354	3.009
	0.3993	0.3878	1.900	1.747	3.381	3.026
	0.583	0.560	1.943	1.787	3.512	3.142
	0.596	0.572	2.037	1.862	3.539	3.165
	0.631	0.604	2.179	1.996	3.801	3.383
	0.686	0.653	2.446	2.233	3.867	3.432
	0.703	0.672	2.457	2.244	4.191	3.695
	0.984	0.925	2.510	2.282	4.318	3.800
	1.050	0 991	2.621	2.381	4.850	4.216
	1.239	1.154	2.727	2.468	5.082	4.413
	1.306	1.218	2.902	2.623	5.155	4.467
	1.422	1.323	3.069	2.770	5.774	4.941

l-Hydroxyproline—Purchased from Hoffmann-La Roche, Inc. Nitrogen calculated, 10.7 per cent; found, 10.8.

dl-Serine—Purchased from Amino Acid Manufactures. Solubility, 0.491 and 0.489 mole per 1000 gm. of water.

dl-Threonine—Purchased from the Department of Chemistry, University of Illinois. Solubility, 201.0 and 201.5 gm. per 1000 gm. of water.

Sarcosine—Purchased from Hoffmann-La Roche, Inc. Solubility, 18.46 and 18.43 moles per 1000 gm. of water.

Betaine—Prepared from betaine chloride purchased from the Eastman Kodak Company. Nitrogen calculated, 11.89 per cent; found 11.75.

It was assumed that the values of the osmotic coefficients of the sucrose used as the reference standard could be represented by the equation

$$\varphi = 1 + 0.084m + 0.0104m^2 - 0.00237m^3 + 0.000115m^4$$

Results

The isopiestic molalities of the sucrose and amino acids are given in Table I. Smoothed values of m_S/m_A were used in the

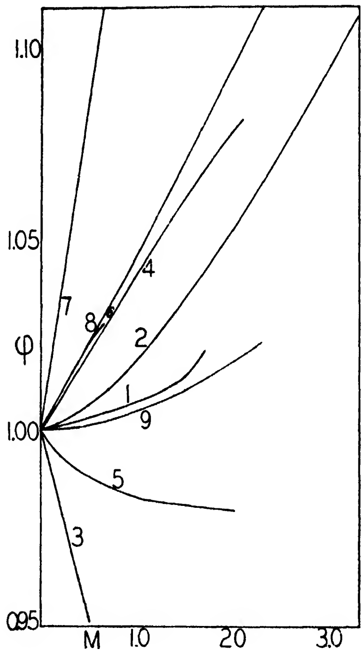

FIG. 1. Curve 1, *dl*-alanine, Curve 2, sarcosine, Curve 3, *dl*-serine, Curve 4, *dl*-α-amino-*n*-butyric acid, Curve 5, *dl*-threonine, Curve 6, *dl*-α-amino-*n*-valeric acid, Curve 7, betaine, Curve 8, proline, Curve 9, hydroxyproline.

calculation of the osmotic coefficients from the relation $\varphi_A = \varphi_S(m_S/m_A)$.

The results are shown in Fig. 1 and are represented, usually within ± 0.001, by the following equations.

dl-Proline—$\varphi_A = 1 + 0.04635m - 0.002009m^2 + 0.001646m^3 - 0.0001904m^4$
l-Hydroxyproline—$\varphi_A = 1 + 0.0007326m + 0.0044m^2 - 0.0001656m^3$

dl-Serine—$\varphi_A = 1 - 0.0854m - 0.0505m^2 + 0.0425m^3$
dl-Threonine—$\varphi_A = 1 - 0.0323m + 0.0185m^2 - 0.0038m^3$
Sarcosine—$\varphi_A = 1 + 0.008847m + 0.009898m^2 - 0.0007366m^3 - 0.000009m^4$
Betaine—$\varphi_A = 1 + 0.17277m - 0.05794m^2 + 0.0005224m^3 - 0.0000341m^4$

The activity coefficients were calculated from these equations as described in the previous paper (12). These data are summarized in Table II.

TABLE II

Activity Coefficients

m	*dl*-Serine	*dl*-Threonine	*dl*-Proline	*l*-Hydroxyproline	Sarcosine	Betaine
0.2	0 964	0.988	1.019	1.001	1.004	1.071
0.3	0.945	0.983	1.028	1.001	1.006	1.108
0 5	0.907	0.974	1.047	1.002	1.012	1.186
0.7		0 967	1.068	1.003	1.020	1.269
1.0		0.959	1.096	1.007	1.032	1.403
1.2		0.955		1 010	1.042	1.499
1.5		0.950	1.148	1.015	1.059	1.654
2.0		0.943	1.206	1.026	1.091	1.945
2.3				1 034		
2.5			1.269		1.128	2.282
3.0			1 338		1.173	2.672
3.5			1.406		1.221	3.091
4.0			1.495		1.275	3.643
5.0			1.672		1.391	3 933
6.0			1.828		1.513	
7.0			1.979		1.627	
7.3			2.002			

DISCUSSION

The substitution of the strongly polar hydroxyl group in the hydrocarbon chain depresses considerably the values for the osmotic coefficients (compare serine with alanine, threonine with α-amino-n-butyric acid, and hydroxyproline with proline). The amino acids in which methyl groups were attached to the nitrogen have much higher osmotic coefficients than do their analogues with straight hydrocarbon chains (compare sarcosine with alanine and betaine with α-amino-n-valeric acid).

A plot of $-\log \gamma/C$ against the ratio of the dielectric constant of the solvent to that of the solutions, D_0/D (the relationship suggested by Cohn, McMeekin, Ferry, and Blanchard (3)), gives

lines that are either straight or have very little curvature (Fig. 2). The values of $-\log \gamma/C$ at infinite dilution are given in Table III, along with those for $-\log N_A/N_0$ derived from solubility measure-

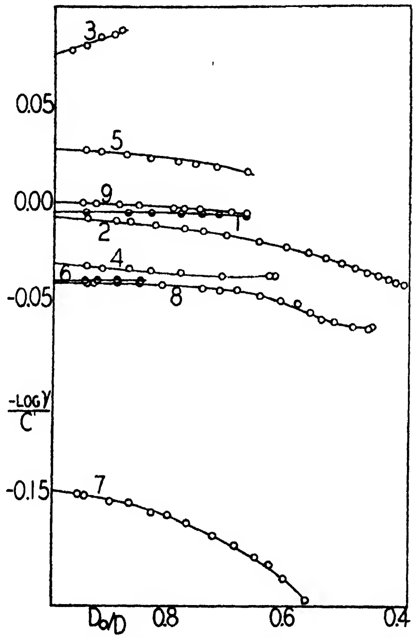

FIG. 2. Curve 1, *dl*-alanine, Curve 2, sarcosine, Curve 3, *dl*-serine, Curve 4, *dl*-α-amino-*n*-butyric acid, Curve 5, *dl*-threonine, Curve 6, *dl*-α-amino-*n*-valeric acid, Curve 7, betaine, Curve 8, *dl*-proline, Curve 9, *l*-hydroxyproline. The values for D_0 and of D for Curves 1, 4, and 6 are from Wyman and McMeekin (15), those for Curve 2 from Devoto (5), those for Curve 7 from Edsall and Wyman (7), those for Curves 8 and 9 from Lindquist and Schmidt (9), while those for Curves 3 and 5 were assumed to be the same as their corresponding non-hydroxy analogues. The partial molal volumes used in calculating C from m for Curves 1 and 4 are from Daniel and Cohn (4), those for Curves 3, 8, and 9 from Cohn, McMeekin, Edsall, and Blanchard (1), those for Curve 7 from Edsall and Wyman (7), and those for Curves 2, 5, and 6 were estimated to be $60.6 + 0.6C$, 76.5, and 92.7 respectively.

ments, and the salting-in and salting-out constants, K_R^* and K_s^*, obtained by the method described in the preceding paper (12). The effect of substitution of a hydroxyl group is to decrease the values for log γ/C and of K_s^*, but, while K_R^* for serine is

much less than that for alanine, the salting-in constants for threonine and hydroxyproline are similar to those for their respective unsubstituted analogues. As with the other amino acids the changes in the values for log N_A/N_0 resulting from substitution of a hydroxyl group parallel in direction those for log γ/C. The amino acids in which methylene groups are added to the nitrogen rather than to the chain have higher values for log γ/C and salting-

TABLE III

Functions Derived from Change of Activity Coefficient with Concentration and Solvent

	$-\frac{\text{Log }\gamma}{C}$ at $\frac{D_0}{D} = 1$	$-\text{Log}\frac{N_A}{N_0}$	K_R*	K_s*
dl-α-Alanine . . .	−0 005	2 856†	0.010	0.015
Sarcosine . .	−0.008		0.016	0.024
dl-Serine	0 075	3.362‡	−0.102	−0.177
dl-α-Amino-*n*-butyric acid.	−0.033	2.375†	0.016	0 049
dl-Threonine .	0.027	3.070‡	0.014	−0.013
dl-α-Amino-*n*-valeric acid	−0.042	(2.158)§	0.000	0.042
Betaine ..	−1.150	0.075‡	0.042	0.192
dl-Proline	−0.043	(1.50)‖	0.004	0.047
l-Hydroxyproline	−0.001	(2.3)¶	0.008	0.009

† Cohn, McMeekin, Edsall, and Weare (2).

‡ Edsall (6).

§ This is the ratio for valine (2). That for α-amino-*n*-valeric acid is probably about 2.00 (6).

‖ From the solubility equation of Tomiyama and Schmidt for aqueous solutions of *l*-proline at 19° (14) and the measurement of Kapfhammer and Eck in alcohol at 19° (8).

¶ From the solubility equation for aqueous solutions at 40° (14) and the solubility of *l*-hydroxyproline in a 0.005 M alcoholic solution of *l*-proline at 40° (8).

in and salting-out constants although those of sarcosine are not much higher. In the one case where a comparison is possible the values for log N_A/N_0 parallel those of log γ/C.

The authors are indebted to Professor Edwin J. Cohn of the Harvard Medical School for helpful suggestions in interpreting these results and to Professor David I. Hitchcock for valuable criticisms.

SUMMARY

Isopiestic vapor pressure measurements have been made at 25° of aqueous solutions of *dl*-proline, *l*-hydroxyproline, *dl*-serine, *dl*-threonine, sarcosine, and betaine.

The osmotic coefficients and activity coefficients of these amino acids have been calculated from the measurements.

Relationships between the activity coefficients and the dielectric constants of their solutions have been discussed.

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THE RELATION OF VITAMIN B_6 AND PANTOTHENIC ACID TO FACTOR W STUDIES*

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Studies on the vitamin B complex requirements of the rat have established the essential nature of three specific factors; *i.e.*, thiamine, riboflavin, and vitamin B_6. The fraction of liver extract which must be supplied in addition to these factors to produce optimum growth has been termed by us factor W (1). That this fraction is multiple in nature has been recognized for some time. Its relation to the other members of the vitamin B complex and to the rat "filtrate factors" of other workers has been discussed in a previous publication from this laboratory (2). In the rations used for the assay of factor W preparations white corn has been the source of vitamin B_6. Since no dermatitis was observed in any of our rats and since no growth response occurred when vitamin B_6 was added either alone or with a crude factor W preparation, we assumed that our basal ration was not deficient in this factor. However, further work has shown that the decreased growth-promoting property of some of our purified fractions of factor W could be eliminated when the fractions were supplemented with synthetic vitamin B_6. Thus the absence of a typical dermatitis is not evidence that the rat is receiving a sufficient supply of vitamin B_6. We have obtained significant growth responses when rats on our basal ration plus purified concentrates of factor W were given additional vitamin B_6 in spite of the fact that the basal ration contained corn, dextrin, and cottonseed oil.

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† Eli Lilly and Company Fellow.

Failure to obtain this response previously was undoubtedly due to the vitamin B_6 present in our factor W preparations.

The ration used for all of the assays reported here, unless otherwise indicated, has the following composition: dextrin 63, casein (Labco) 18, Salts III (3) 4, white corn 6, cottonseed oil 2, cod liver oil 2, butter fat 5, vitamin B_1 150 micrograms, riboflavin 300 micrograms, and nicotinic acid 2.5 mg. When we discovered the vitamin B_6 deficiency in the ration, 300 micrograms of crystalline vitamin B_6 per 100 gm. of ration were added.[1] The assays made when this supplement was included are marked with an asterisk in Table I. Male rats, 21 days old, placed on this ration with or without vitamin B_6, grow to a weight of 70 to 90 gm. in 3 to 5 weeks and are used for assay when their growth rate is reduced to 6 gm. per week or less. Responses of about 28 gm. per week are obtained over a 5 week period when such rats are fed 0.2 gm. of liver extract per day. When purified preparations are fed, responses of 15 gm. per week over a 3 week period are considered significant.

In our early work (4) it was shown that concentrates of factor W did not contain sufficient amounts of the chick antidermatitis factor to prevent dermatitis in chicks. Recently evidence has been brought forth (5-8) indicating that the chick antidermatitis factor, which is probably identical with pantothenic acid, is essential in the diet of the rat. In this paper we wish to present evidence that our improved preparations of factor W still contain considerable pantothenic acid and that part of the growth-promoting activity of these preparations is due to the presence of this vitamin, but that they also contain at least one other factor (factor W) which is required in addition to pantothenic acid for normal growth.

Methods

A new procedure based largely upon properties previously reported (1,2,4) has been adopted for obtaining purified preparations of factor W. The procedure, outlined in Diagram 1, is as follows: 5 kilos of 95 per cent alcohol-soluble liver extract[2] are extracted twice by being stirred with 10 liters of n-butanol. Only

[1] We are indebted to Dr. D. F. Robertson of Merck and Company, Inc., for generous supplies of thiamine, nicotinic acid, and vitamin B_6.

[2] Kindly supplied by Dr. David Klein, The Wilson Laboratories, Chicago.

TABLE I

Growth Responses of Rats Fed Various Fractions of Liver Extract

Experiment No.	Description of preparation	Equivalent of liver extract given daily	Solid material given daily	Pantothenic acid in rat day dose	No. of rats	Duration of experiment	Average wkly. growth
		gm.	*mg.*	*micrograms*		*wks.*	*gm*
1	Control, no supplement				2	4	3- 6
	" liver extract	0.2		60–100	2	5	27–28
2	Hexane-butanol filtrate	0.2	5.0–8.0	30– 32	3	5	21
3	Alkaline norit "	0.2	3.0–4 5		2	5	19
4	Butanol eluate of norit	0.1	1.1		1	5	18
		0.4	4.4		2	5	20
5	Acetone " " "	0.2	1.0	35	2	4	14*
		0.4	2.0	70	2	4	20*
	Butanol " following acetone elution	0.2			1	3	6*
		0.8			1	3	22*
	Acetone eluate + following butanol eluate	0.2			2	3	18*
6	Ethyl acetate eluate of norit	0.2	2.3		1	5	16
7	Fullers' earth filtrate of hexane-butanol filtrate	0.2			3	5	13
	Butanol eluate of fullers' earth	0.2			2	3	5
	Fullers' earth filtrate + eluate	0.2			3	3	22
8	Phosphotungstic acid-butanol filtrate	0.2	0.62		1	4	9
		0.8	4.9		1	4	11
	Same + crystalline vitamin B_6	0.2	0.62		2	3	23*
	Crystalline vitamin B_6, 3 micrograms per gm. ration				2	2	6
9	Chloroform extract of brucine salts	0.2		30	2	4	15*
	Chloroform residue of brucine salts	0.2		45	2	4	19*
	Aqueous extract of brucine salts from chloroform	0.2		30	1	4	16*
10	Acetate-ether filtrate	0.5			2	3	19*
	" precipitate	0.5			2	2	2*

* Assayed with crystalline vitamin B_6 in the ration.

a small amount of activity remains in the insoluble residue from which the extract is decanted. To the clear butanol solution is added an equal volume, 22 liters, of hexane (Skellysolve B), which precipitates a large amount of inactive material. After the solution has stood for several hours to allow the precipitate

Diagram 1

Steps in Concentration of Factor W

95 per cent alcohol-soluble liver extract
(extract with *n*-butanol)

Residue (discard)	Butanol solution (add equal volume of hexane)
Precipitate (discard)	Hexane-butanol filtrate (concentrate *in vacuo* and take up in water)
Water-insoluble residue (discard)	Aqueous solution (adjust pH to 12.0 and treat with norit) (Experiment 2)
Adsorbate (discard)	Alkaline norit filtrate (adjust pH to 6.0 to 7.0 and treat five successive times with norit) (Experiment 3)
Norit filtrate (discard)	Norit adsorbate (dry at 60° overnight and elute four successive times with boiling butanol)
Eluted norit (discard)	Butanol eluate (add butanol solution of phosphotungstic acid) (Experiment 4)
Precipitate (discard)	Phosphotungstic acid-butanol filtrate (concentrate *in vacuo* and take up in water. Neutralize with saturated $Ba(OH)_2$)
Barium phosphotungstate (discard)	Filtrate (Experiment 8)

to settle, the clear filtrate is decanted and the solvents removed by distillation under reduced pressure. The solid extract is stirred with 1 liter of water and the water-insoluble portion removed on filter-cel. This fraction is very active when fed at low levels. The solution is diluted to 5 liters with water and adjusted to pH 12.0 with concentrated NaOH. The solution is then treated

with 200 gm. of norit A (Pfanstiehl) for 30 minutes, and the norit filtered off with suction. Nearly all of the activity is in the filtrate. The solution is adjusted to pH 6.0 to 7.0 with dilute sulfuric acid and treated five successive times with 100 gm. portions of norit for 30 minute periods. The norit is filtered off and dried at 60° overnight, and the inactive filtrate is discarded. The active material may be eluted from the norit by being refluxed three or four times with any of a number of solvents. We have found *n*-butanol to be most satisfactory, but have also used dry acetone. The acetone removes only part of the activity and the remainder of the activity is removed by butanol. The elution with the two solvents does not give a separation of factors, since the results in Table I show no supplementary effect between the two fractions. The butanol eluate is concentrated to a volume such that 1 cc. is equivalent to 1 gm. of the original liver extract. To this butanol solution is added 0.5 volume of a saturated butanol solution of phosphotungstic acid. The heavy precipitate is filtered off and washed with butanol, and the filtrate and washings concentrated under reduced pressure and taken up in water. A saturated solution of barium hydroxide is used to precipitate the excess phosphotungstic acid. The filtrate is active at levels considerably below 1 mg. per rat per day when fed with vitamin B_6, but is quite inactive in the absence of this factor. Typical growth responses are shown in Table I.

During the course of the procedure some attempt was made to follow the active compound or compounds by means of color reactions and standard qualitative tests. Samples of many of the preparations were coupled, in alkaline solution, with diazotized sulfanilic acid to give a very distinct orange color. However, the phosphotungstic acid-butanol filtrate gives no such color reaction though it is very active when fed to the rat. Kuhn and Löw (9) have developed a similar test for vitamin B_6, and crystalline vitamin B_6 produced an orange color with our reagent. Hence, it is very probable that vitamin B_6 is the compound we detected, for our more purified concentrates which did not give this test were not active unless fed with vitamin B_6.

The cyanogen bromide reaction, which is widely used in the determination of nicotinic acid, was positive on the phosphotungstic acid-butanol filtrate. Kuhn and Löw (9) have shown

that vitamin B_6 also gives a color with this reagent but, since this preparation contains no vitamin B_6, the color is probably due to some other pyridine derivative, presumably nicotinic acid.

The ferric chloride test for phenols and enols and Bial's test for pentoses were negative on the more purified preparations. Other qualitative tests which are completely negative on some of the active concentrates are the Molisch test for carbohydrates, the ninhydrin test for amino acids, and the Ehrlich and Hopkins-Cole reactions for indole derivatives.

By the Fiske-Subbarow method there appeared to be less than 1 microgram of inorganic phosphorus per rat day dose of an active preparation. Micro-Kjeldahl determinations indicated a nitrogen content for several of the concentrates varying from 2.9 to 15.3 per cent.

Fullers' Earth Fractions—100 cc. of an aqueous solution of the hexane-butanol filtrate fraction equivalent to 50 gm. of original liver extract, adjusted to pH 1.0 with dilute sulfuric acid, are treated for three 30 minute periods with 3 gm. portions of English fullers' earth. The fullers' earth is washed with 0.1 N H_2SO_4 and the washings added to the filtrate. After the fullers' earth is dried at 60° overnight, it is refluxed three successive times with *n*-butanol.

As shown in Table I, the filtrate fraction alone and the eluate fraction alone produced small growth responses, but when the two were fed together a very definite supplementary effect was obtained.

Acid-Ether Extraction—An aqueous solution of the hexane-butanol filtrate fraction, adjusted to pH 1.0 with dilute sulfuric acid, is extracted continuously for 72 hours with peroxide-free ethyl ether. We have considerable evidence that the residue and extract supplement each other. Typical results are shown in Fig. 1. Because white corn is known to contain pantothenic acid, one series of assays was made without corn in the ration. These results are shown in Table II.

Formation and Extraction of Brucine Salts—To an aqueous solution of the hexane-butanol filtrate fraction an alcoholic solution of brucine is added in an amount necessary to neutralize the acidity of the solution. A large amount of filter-cel is then added, and the solution evaporated to dryness before a fan at 60°, with

occasional stirring. The dried material is extracted by being refluxed twice with chloroform. The chloroform is removed under reduced pressure and the extracted material taken up in a small amount of water. The chloroform-insoluble portion is also taken up in water, and both fractions are freed of brucine by addition of a barium hydroxide solution. After the brucine is filtered off, the free acids are liberated and the barium precipitated with dilute sulfuric acid. A 100 cc. portion of the chloroform solution is extracted by being shaken with two 10 cc. portions of water and the aqueous solution freed of brucine as described above.

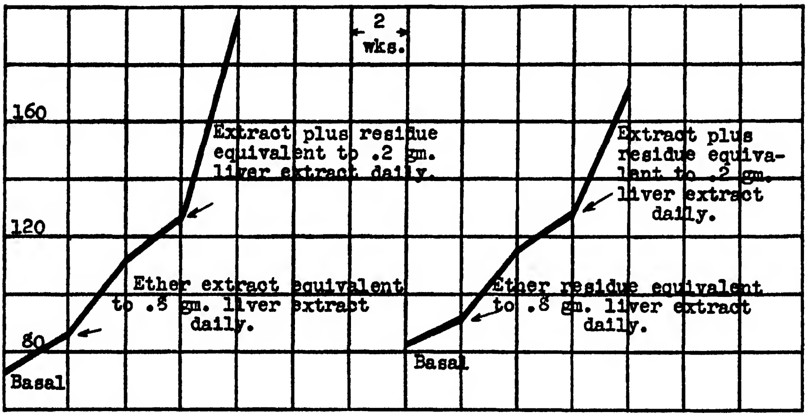

FIG. 1. Growth curves showing the supplementary effect of acid-ether extract and residue.

As shown in Table I, active concentrates were prepared by use of the brucine salts, but the greater portion of the activity remained in the chloroform residue. All of the active material in the chloroform extract was transferred from the chloroform to the aqueous phase.

Formation of Acetate—A portion of the hexane-butanol filtrate fraction equivalent to 50 gm. of the original liver extract is evaporated to dryness under reduced pressure and taken up in 25 cc. of redistilled pyridine. 75 cc. of acetic anhydride are added and the reaction mixture allowed to stand at room temperature for 1 week. The mixture is then reduced to a volume of 5 cc. by distillation under reduced pressure and is added to 15 cc. of

ethyl ether. The resulting precipitate is allowed to settle and the supernatant liquid decanted.

The precipitate is taken up in 0.1 N NaOH and allowed to stand at room temperature overnight, when the solution is neutralized with sulfuric acid. The ether is removed from the filtrate under reduced pressure and the residue hydrolyzed by the method used for the precipitate. The hydrolyzed precipitate was completely inactive, and the filtrate contained less than one-half of the original activity (Table I).

Pantothenic Acid Assays[3]—Several of our preparations were assayed for their content of pantothenic acid by the method of Snell, Strong, and Peterson (10), in which the organism *Lactobacillus casei* is used. The pantothenic acid content of the fractions assayed is shown in Table I. The assays show that we were able to extract only a portion of the brucine pantothenate with chloroform, although our method for the formation and extraction of brucine salts is similar to that of Williams *et al.* (11) for the concentration of pantothenic acid.

TABLE II

Experiment 11. Growth Responses of Rats Fed Acid-Ether Extract and Residue

The assays shown in this table were made on a ration similar to the one described except that the white corn is replaced with an additional 6 per cent of dextrin.

Two rats were each given the equivalent of 0.2 gm. of liver extract daily for 2 weeks in tests for each preparation.

Description of preparation	Pantothenic acid in rat day dose	Average wkly. growth
	micrograms	*gm.*
Acid-ether extract of hexane-butanol filtrate..	50	8
" residue " " " ..	0	2
" extract + residue	50	19

DISCUSSION

It is quite evident from these data that some of our results with purified concentrates of factor W have been complicated by an inadequate supply of vitamin B_6. In our earlier work (2) we

[3] Mr. E. J. Stoltz made these assays.

pointed out the possibility that our factor W concentrates could overcome a partial lack of vitamin B_6, and that greater growth could be obtained than that attributable to factor W addition alone. We also reported (1) an apparent separation of two factors by precipitation with mercury and barium, and from a consideration of its properties this may have been an incomplete separation of vitamin B_6 from factor W. However, it is also possible that factor W and pantothenic acid were separated by barium precipitation in an alcohol solution. A destructive effect upon the activity of factor W concentrates when treated with fullers' earth has also been previously noted (12). We now believe that this effect is due to adsorption of vitamin B_6 on the fullers' earth. The results in Table I indicate a very definite supplementary effect between the fullers' earth filtrate and the butanol eluate of fullers' earth. We believe the activity of the eluate is due to vitamin B_6. Our phosphotungstic acid-butanol filtrate is probably more nearly free of vitamin B_6 than any other fraction we have prepared. That it produces a good growth response only when fed with crystalline vitamin B_6 is our best evidence that our ration is deficient in this factor, and that it is required in greater amounts for optimum growth than for prevention of dermatitis. Because the addition of vitamin B_6 produces no response on our ration, we believe that it does not become a limiting factor in the ration until factor W is included, and that with vitamin B_6 included, the ration is satisfactory for the assay of factor W preparations.

In 1937 Halliday and Evans (13) reported results which indicated that the response attributed to factor W may be due to vitamin B_6. We believe it is now evident that part of our response was due to vitamin B_6, and that the excellent growth of their animals was due to both vitamin B_6 and factor W in their preparations.

Recently several workers (5–8, 14) have reported evidence for the essential nature of pantothenic acid in the diet of the rat. Though final proof awaits the isolation of this factor, our results confirm this evidence. Of the fractions assayed for pantothenic acid none which was low in this factor produced a normal growth response. Our best evidence for its essential nature is the supplementary effect of the acid-ether extract and residue, shown in Fig. 1 and in Table II. The extract contains a considerable

amount of pantothenic acid, while the residue has none. We are unable to explain our previous failure to effect a separation of factors by acid-ether extraction, except that those results may have been complicated by the vitamin B_6 deficiency. Acetylation and formation of brucine salts are steps which have been used in the concentration of pantothenic acid. That we can use these procedures for the preparation of concentrates which are active in promoting rat growth is evidence that at least one of the essential factors is very similar in its properties to pantothenic acid. Hitchings and Subbarow (5,14) have also used an acid-ether extraction and formation of brucine salts as steps in concentrating a factor essential for rat growth. Hoffer and Reichstein (6) have previously reported a supplementary effect between the acid-ether extract and residue, and El-Sadr *et al.* (7) have successfully acetylated active preparations.

The question of nomenclature needs further consideration. Originally factor W was considered to be a single factor, essential for rat growth in addition to thiamine, riboflavin, and vitamin B_6. Later it became evident that it was multiple in nature. We have now separated two factors by an acid-ether extraction. Because the factor in the ether extract is probably pantothenic acid, a known compound, we believe that the term factor W should be reserved for the factor in the residue. The greater growth response given by liver extract over the hexane-butanol filtrate (Table I), which contains both pantothenic acid and factor W, suggests that another factor, in addition to pantothenic acid and factor W, may be essential in the nutrition of the rat.

SUMMARY

1. A procedure for the preparation of highly purified factor W concentrates is described.

2. Evidence is presented indicating that the factor W assay ration previously used is deficient in vitamin B_6. Significant growth responses are obtained when synthetic vitamin B_6 is added to our basal ration plus factor W concentrates.

3. All factor W preparations which promote normal rat growth contain significant amounts of pantothenic acid.

4. Evidence is presented to show that these concentrates con-

tain at least one other growth factor for which the term factor W is retained.

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INHERITANCE EFFECT OF EXERCISE ON THE PHOSPHOLIPID AND CHOLESTEROL CONTENT OF MUSCLE

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As the result of examination of the phospholipid and cholesterol content of various tissues in animals, it was found that a high lipid content was associated with activity. The relation seemed to be especially well marked in muscle and an examination of the lipids of voluntary muscle in various animals showed wide differences in phospholipid and cholesterol content which could be associated with varying extents of natural muscular activity (1). An experimental study of the effect of exercise on muscle lipid was then made (2) in the hope of explaining some of the very wide differences in the lipid content of the same muscles found in different animals. By exercising one group of rats in an exercising cage and resting a control group by confinement, it was found that the experimental activity produced in the exercised muscles a higher content of phospholipid with a less marked increase of cholesterol and a resulting higher phospholipid to cholesterol ratio. These differences with exercise while characteristic were not invariable and not great enough to serve as an explanation of the large differences found in nature. For example, it was found that the leg muscles of certain wild mice had more than twice the percentage of phospholipid and nearly twice as much cholesterol as the corresponding muscles in the laboratory rat.

In seeking an explanation for the difference, we considered the factor of heredity in the sense of great activity through several generations and put this theory to experimental test as follows: Female rats which had run well in the exercising cage for a month or longer were removed from the cage and bred. The offspring, when they had reached the experimental weight (about 120 gm.),

were put into the cage and similarly exercised, after which some were killed for analysis and a certain number were bred. Their daughters, when they reached the experimental weight, were allowed to run for the usual period (1 to 2 months), and were then killed, and the weighed muscles analyzed for phospholipid and cholesterol. Eight members of the second generation and four of the third were examined. The lipid content of the muscles of the second generation was markedly higher than that of the first generation, while that of the muscles of the third generation was only slightly different from that of the second. For this reason, the values for these two generations will be considered together. There were twelve animals in this group as compared with fifteen each in the first resting and exercised groups.

The muscles or muscle groups used were the heart, neck, diaphragm, abdominal wall, back and loin, thigh, gastrocnemius, front leg, and pectoralis. Because of the difficulty in getting comparable samples of neck muscles and because of the relatively small changes in composition, this muscle group is omitted from the present discussion. The treatment of the muscle samples and the analyses were carried out as in the previous report; *i.e.*, removal of the muscle as soon as possible after death, grinding with sand, and extraction with hot alcohol. Phospholipid was determined in the extract by the oxidative method after precipitation with acetone and magnesium chloride, and the cholesterol was determined colorimetrically. The results for the group as average values in terms of moist weight with standard deviations and percentage changes are given in Table I which contains also, for comparison, the resting and exercise values for the first generation obtained in the earlier work.

Results

The later generations differed from the first chiefly in that the effects noted were much more marked, although in the same direction. The changes in values for the second and third generations in terms of the resting values for the first generation were as follows:

Hypertrophy—Over 50 per cent, back and loin 92, heart 68, pectoralis 60, gastrocnemius 53; 30 to 50 per cent, abdominal wall 37, diaphragm 36; below 30 per cent, front leg 21; diminished, thigh 56.

TABLE I

Inherited Effect of Exercise on Muscle Lipid

Muscle	Condition	Muscle weight, per cent body weight			Phospholipid, per cent moist weight			Cholesterol, per cent moist weight		
		Average	Standard deviation	Change	Average	Standard deviation	Change	Average	Standard deviation	Change
Heart	Resting	0.302	±0.02		1.68	±0.17		0.119	±0.01	
	Exercised, I*	0.352	±0.03	+16.5	1.94	±0.146	+15.5	0.135	±0.032	+7.4
	" II and III*	0.508	±0.083	+68.2	1.67	±0.35	−0.6	0.156	±0.05	+31.1
Diaphragm	Resting	0.260	±0.03		1.05	±0.1		0.091	±0.01	
	Exercised, I	0.361	±0.19	+38.5	0.92	±0.16	−14.1	0.077	±0.02	−18.2
	" II and III	0.354	±0.052	+36.2	1.12	±0.214	+6.7	0.148	±0.056	+62.6
Thigh	Resting	6.41	±1.1		0.84	±0.106		0.064	±0.003	
	Exercised, I	7.24	±1.3	+12.9	0.87	±0.09	+3.6	0.060	±0.01	−6.6
	" II and III	4.12	±0.86	−55.5	0.97	±0.1	+15.3	0.088	±0.019	+37.5
Front leg	Resting	2.18	±0.7		0.89	±0.096		0.072	±0.01	
	Exercised, I	2.53	±1.05	+16.1	1.03	±0.085	+15.7	0.077	±0.01	+6.9
	" II and III	2.63	±0.76	+20.6	1.09	±0.3	+22.5	0.111	±0.027	+54.2
Abdominal wall	Resting	3.39	±0.3		0.73	±0.09		0.066	±0.03	
	Exercised, I	3.66	±0.6	+8.0	0.77	±0.1	+5.5	0.071	±0.01	+7.6
	" II and III	4.64	±0.8	+36.9	0.84	±0.121	+15.2	0.082	±0.017	+24.4
Gastrocnemius	Resting	1.09	±0.2		0.85	±0.09		0.068	±0.009	
	Exercised, I	1.32	±0.3	+21.1	0.97	±0.04	+4.1	0.076	±0.003	+11.8
	" II and III	1.67	±0.33	+53.2	1.09	±0.21	+28.2	0.119	±0.05	+75
Back and loin	Resting	2.51	±0.7		0.73	±0.09		0.059	±0.011	
	Exercised, I	2 96	±0.6	+17.9	0.82	±0.09	+12.3	0.062	±0.01	+5.1
	" II and III	4.81	±1.4	+91.6	0.85	±0.12	+16.2	0.091	±0.019	+54.2
Pectoralis	Resting	0.918	±0.14		0.89	±0.13		0.074	±0.01	
	Exercised, I	1.00	±0.2	+8.9	0.97	±0.1	+9	0.077	±0.01	+4.1
	" II and III	1.47	±0.32	+60.1	1.07	±0.23	+21.3	0.105	±0.04	+41.8

* I = first generation; II = second generation; III = third generation.

Increased Phospholipid—Over 20 per cent, gastrocnemius 28, front leg 23, pectoralis 21; 10 to 20 per cent, back and loin 16, abdominal wall 15, thigh 15; below 10 per cent or negative, heart and diaphragm.

Increased Cholesterol—Over 50 per cent, gastrocnemius 75, diaphragm 63, back and loin 54, front leg 54; 25 to 50 per cent, pectoralis 42, thigh 37, heart 31; below 25 per cent, abdominal wall.

The compensatory changes or responses to the exercise were one or all of the following: (*a*) hypertrophy in all but the thigh muscles, (*b*) increased cholesterol in all the muscles, (*c*) increased phospholipid in all the muscles but the heart and diaphragm. Thus the change in the heart was mostly in increased size with a less important increase of cholesterol. In the gastrocnemius, the greatest increase was in the cholesterol content, with a good increase in phospholipid and a less marked hypertrophy. The muscles of the front leg showed relatively little hypertrophy but a good increase in phospholipid and cholesterol, while the pectoral muscles showed marked increases in all three.

Hypertrophy, as shown by relation of muscle weight to body weight, was much more pronounced in the later generations than in the first. For example, in the back and loin the increase in weight was 92 per cent in the later generations as compared with 18 per cent in the first, in the pectoralis 60 per cent as compared with 8 per cent, 68 per cent as compared with 16 per cent in the heart. The diaphragm was an exception. It showed 36 per cent hypertrophy in the first generation and none in the later ones.

The increase in phospholipid was significant and considerable but not great in all muscles except the heart and diaphragm and notably greater in the later generations than in the first. The increase was greatest in the front leg, the pectoralis muscles, and the gastrocnemius. The increase in cholesterol in the second group was very great and of the same order as the hypertrophy. It took place in all the muscles without exception and was much greater than in the first group.

Certain muscles, which can be definitely related to the type of exercise employed (running on a curved surface), show especially marked changes. For example, the gastrocnemius showed a 53 per cent hypertrophy, a 75 per cent increase in cholesterol, and a 28 per cent increase in phospholipid. The front leg muscles, while not greatly hypertrophied (21 per cent), showed a cholesterol

increase of 54 per cent and a phospholipid increase of 23 per cent. The pectoral muscles were 60 per cent hypertrophied, and contained 21 per cent higher phospholipid than the resting controls and a 42 per cent higher cholesterol. The greatest increases in phospholipid were found in these muscles. The heart was considerably larger (68 per cent), but its cholesterol increase was only 31 per cent and its phospholipid content unchanged. The heart contains normally a much higher percentage of phospholipid and cholesterol than the voluntary muscles.

The muscles least affected by the exercise were those of the abdominal wall and the thigh, the latter weighing less in the later generations than in the first. However, as already noted, the gastrocnemius was one of the most changed of the muscles and it is probable that most of the effort of the type of exercise used fell on the leg rather than on the thigh muscles.

As already noted, next to hypertrophy, the most marked effect of the inheritance was the increase in cholesterol, which was over 50 per cent in four of the eight muscle groups and over 30 per cent in three others. The significance of these high values is not known but attention has been called (1) to the association of high cholesterol with spontaneous or automatic rhythmic activity such as is found in the heart and in smooth muscle. The exercise which these animals took consisted of a monotonous and rhythmic repetition of the same movements by the same muscles which would have therefore much of the character of automatic movement. The fact that cholesterol is an excellent dielectric may have something to do with the continuous accumulation and discharge of nervous (electrical?) energy which would be necessary in automatic or continuous rhythmic movement.

As compared with the results in the first group of animals, the differences shown in the later groups are large enough and enough greater than the standard deviation to be significant and to represent definite differences presumably due to the exercise. As such, the hypertrophy is characteristic and is of course well recognized as an effect of work on muscle. The great increase in cholesterol as the result of exercise has not been noted before and its significance is not known. The values approach those found in animals with very high muscle cholesterol values found in instances mentioned in a previous communication (1). The phos-

pholipid, although considerably increased, does not approach the value found in the muscles of some of the previously mentioned animals. For example, the wild mouse had a phospholipid value of 1.68 per cent in its thigh muscles as compared with the value of 0.97 per cent which is the average value for the thigh muscles of the exercised rats in the second group above. However, as far as the limited numbers allow, the results indicate that the lipid content of muscle may be increased by exercise through succeeding generations.

SUMMARY

Muscles of rats exercised through the second and third generations show, in addition to hypertrophy, large increases in phospholipid and especially in cholesterol. These results are favorable to the idea that exercise through several generations may result in increased phospholipid and cholesterol content of muscle.

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STUDIES ON COLLOIDAL SULFUR-POLYSULFIDE MIXTURE

ABSORPTION AND OXIDATION AFTER ORAL ADMINISTRATION

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Relatively little research has been done on the absorption of elementary sulfur from the gastrointestinal tract. Wild (1) observed that when powdered sulfur was fed most of it passed through the bowel unchanged. Denis and Reed (2) determined inorganic and ethereal sulfate and total non-protein sulfur on animals fed a standard diet to which was added powdered sulfur in a dosage of 500 mg. per kilo per day, and obtained evidence that about 10 per cent of the administered sulfur was absorbed. In view of the great insolubility of sulfur they considered the fraction absorbed to arise from H_2S production by action of the intestinal flora. The absorbability of colloidal sulfur from the intestine was investigated by Maillard (3). He gave rabbits a colloidal sulfur solution prepared by treatment of H_2SO_3 with H_2S and reported a recovery of about 80 per cent of the sulfur in the urine within 24 hours, of which over half appeared in the unoxidized form. Because of the recent fairly wide-spread use of colloidal sulfur as a therapeutic drug, it was considered advisable to corroborate and extend this evidence.

According to the prevailing opinion the sulfur compounds of the urine, consisting chiefly of inorganic sulfate, are non-threshold bodies and do not remain in the circulation. Thus Denis and Reed (4) found that following introduction of H_2S into the intestine there was no marked increase in blood sulfate level unless the kidneys were ligated. It follows that in the absence of renal pathology accurate information regarding sulfur excretion may be obtained by appropriate urine analyses. It is also essential

to take into account the sulfur normally present in the urine as an end-product of protein metabolism; this may be done by nitrogen determinations.

In investigating the toxicity of a colloidal sulfur preparation which is a concentrate of a natural sulfur spring,[1] it was found that when this was given orally in a sufficiently large dose intoxication and death occurred within 5 minutes (5). This was strong presumptive evidence that absorption was prompt. It was also noted that the toxic dose was very large, that the material was innocuous when fed over long periods of time to rats, rabbits, and dogs in doses up to 80 mg. per kilo, and that it could be given to human subjects with perfect safety in amounts as high as 750 mg. Therefore two series of experiments were conducted, in the first of which six individuals were maintained on a constant diet for a 10 day period comprising an initial 3 day control régime followed by 7 days in which a given amount of sulfur was ingested; the nitrogen and sulfur excretion was followed by daily urinalyses. In the second experiment a single dose of sulfur was administered and sulfur and nitrogen determined on the urines collected every 2 hours for 24 hours. In the latter experiment comparative studies were made on powdered sulfur.

EXPERIMENTAL

Six graduate students served as subjects in the first set of experiments. They were allowed to select their own diet, with the stipulation that the same amount of each food be consumed daily, and 24 hour urine specimens were exactly and quantitatively collected daily. The dietary items were weighed and ingested at a table in our laboratory. After a 3 day control period four of the subjects were given 500 mg., and two of them 750 mg. of colloidal sulfur orally, daily for the remaining 7 days, except in the case of one subject who stopped after 5 days to permit a 2 day after period. Urine nitrogen was determined by the Scales and Harrison (6) modification of the Kjeldahl method, total sulfate by the method of Folin (7), and total sulfur by the Denis (8) modification of the Benedict (9) method.

In the second experiment nine graduate students served as subjects. A 2 hour control urine specimen was collected, 500 mg.

[1] Graham Springs, Kentucky.

of colloidal sulfur or powdered sulfur taken by mouth, and eleven subsequent urine specimens were collected and analyzed as before.

Results

Additions of Colloidal Sulfur to Constant Diet—There was a marked increase in urinary sulfur in the first 24 hour specimen collected after the sulfur ingestion, and sulfur excretion continued high as long as colloidal sulfur was given. Recovery was usually quantitative in the form of sulfate. A representative protocol of one of the six subjects is given in Table I.

TABLE I

Daily S and N Output of Subject J. W. on Constant Diet, Showing Effect of Sulfur Supplement

Day	Total N	Total SO_4-S	Total S	S:N ratio
	gm.	*mg.*	*mg.*	
1	13.1	848	1025	0.0784
2	12.5	683	885	0.0734
3*	9.9	689	867	0.0875
4	12.7	1401	1650	0.1300
5	10.4	1230	1470	0.1412
6	14.2	1590	1810	0.1275
7	10.3	1162	1294	0.1256
8	15.0	1610	1782	0.1190
9	10.0	1105	1310	0.1310
10	14.3	1460	1717	0.1200

* 500 mg. of colloidal sulfur daily at this point.

Rate of Excretion after Single Dose of Colloidal Sulfur—In this series of experiments a very marked increase in sulfur excretion was obtained, maximal in the first or second 2 hour specimen collected after sulfur ingestion. The sulfur content of subsequent 2 hour specimens gradually tapered off to the control level. Typical examples are shown in Figs. 1 and 2 of the urinary sulfur output in eight subjects given colloidal sulfur and powdered sulfur on different days.

DISCUSSION

It is evident that any procedure for the estimation of extra sulfur in the urine must take into account the sulfur formed from

cystine and methionine catabolism, and, furthermore, that the possibility of increased endogenous protein breakdown must be ruled out. Urinary nitrogen determinations and calculation of the sulfur-nitrogen ratio are necessary to make the results of such studies valid. Folin (10) determined the normal sulfur-nitrogen

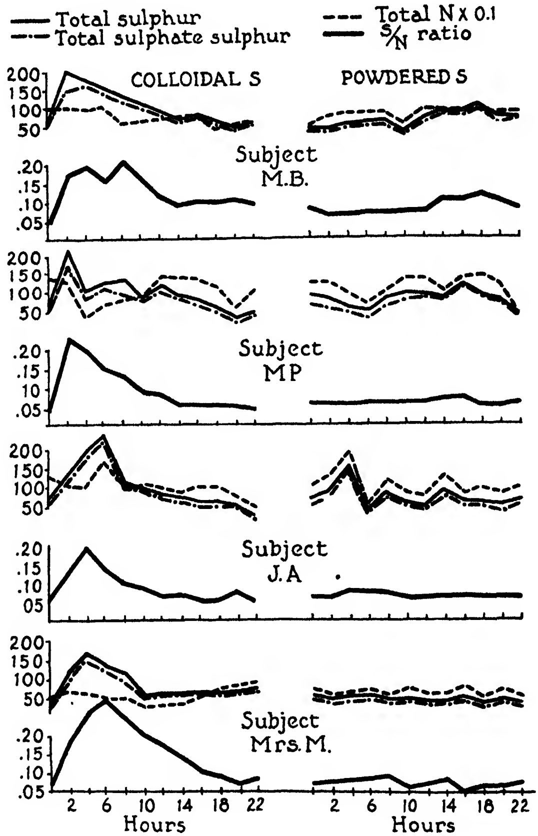

Fig. 1

ratio and its variations in various levels of protein intake, and the control values in the above experiments are in accordance with his data, although we have found a somewhat wider range. For practical purposes, it has been assumed that a sulfur-nitrogen ratio in excess of 0.1 indicates active absorption of elementary sulfur.

In the first series of experiments detailed above it is apparent that when a constant diet is supplemented with sulfur there is a marked rise in urinary sulfur in the first specimen collected after initiation of the sulfur régime. During the control period there was a rough parallelism between nitrogen and sulfur output, and

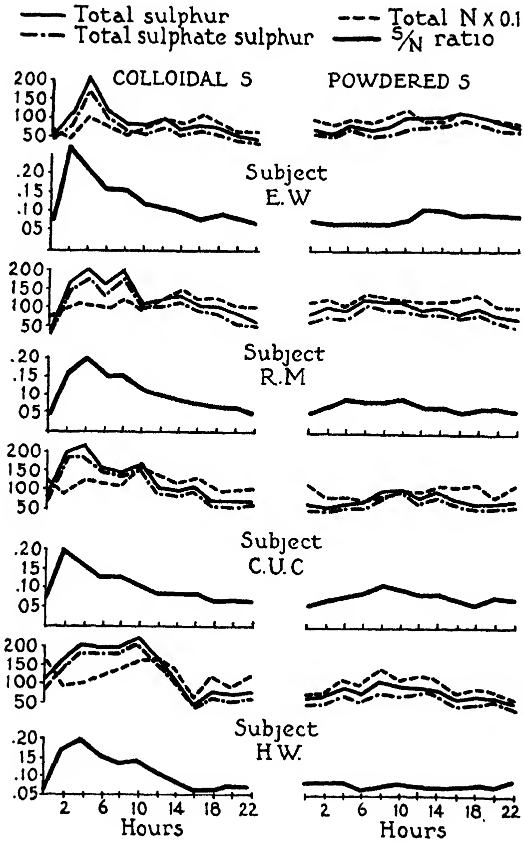

Fig. 2

after the sharp rise in sulfur excretion the parallelism persisted at a new level. The sulfur increase could be accounted for almost entirely by the rise in sulfate output, indicating complete oxidation of the sulfur administered. In the case of the one subject in which a 2 day after period was studied after 5 days of sulfur,

the sulfur excretion returned to normal in the first specimen collected; thus the sulfur is cleared from the system within 24 hours. When the nitrogen and sulfur values for the control and sulfur periods were averaged and compared, it became manifest that sulfur recovery in the urine was in most cases quantitative, as shown in Table II.

The quantitative elimination of sulfur in the urine was verified by a determination of the fecal sulfur during the course of the experiment, and this was found to show no significant variations.

The findings obtained in the second set of experiments are self-explanatory. In every case the administration of colloidal sulfur

TABLE II

Average Daily N and S Output on Constant Diet, Showing Amount of Added Sulfur Recovered

Subject	S daily	Total N		Total SO_4		Total S		S:N ratio		Extra S	Recovery
		Control	S	Control	S	Control	S	Control	S		
	mg.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*			*mg.*	*per cent*
H. B. I.	750	10.7	11.9	818	1549	959	1747	0.090	0.155	788	105
E. W.	500	10.9	11.2	649	1160	810	1309	0.074	0.117	499	100
R. J. G..	750	16.1	16.4	1171	1773	1311	2045	0.081	0.125	734	98
H. W.	500	9.5	9.0	586	869	701	1036	0.062	0.115	335	67
J. W.	500	11 8	12.4	741	1365	925	1433	0.079	0.116	508	102
M. B.	500	6.4	5.8	371	751	496	912	0.079	0.157	416	83

caused a very sharp rise in sulfur output in the first specimen collected after the control, reaching a maximum in 2 to 4 hours and gradually falling off to normal in 14 to 20 hours. In all cases when the sulfur excretion was large there was an increased output of unoxidized sulfur; thus a small portion—probably less than 10 per cent—of the colloidal sulfur ingested escaped oxidation. The substitution of powdered sulfur (100 mesh) for colloidal sulfur showed uniformly that only a small portion of it is absorbed; such absorption as takes place was found to occur at 8 to 16 hours after ingestion, at which time it was probably in contact with the intestinal flora of the lower bowel.

SUMMARY

Orally administered colloidal sulfur in doses of 500 to 750 mg. is completely absorbed, oxidized, and excreted in the urine as sulfate, where the oral dose may be recovered quantitatively in most cases. The absorption and elimination are very rapid, as reflected in a marked increase in urinary sulfate within 2 hours.

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CRITERIA FOR PURITY OF CHLOROPHYLL PREPARATIONS*

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It is inevitable that the separation in pure state of substances which cannot be purified by recrystallization or by other common methods should prove difficult, and that criteria of purity should be correspondingly less convincing. The primary purpose of this paper is to establish satisfactory criteria for several chlorophyll preparations, especially with reference to their absorption of light. This is of fundamental importance in assays of pigment mixtures by spectroscopic means. In recent years, absorption coefficients for the two chlorophyll components have been determined by Winterstein and Stein (1), Zscheile (2), Hagenbach, Auerbacher, and Wiedemann (3), and Meyer (4). The values reported in "Tabulæ biologicæ" (5) do not indicate the startling lack of agreement which is revealed by the most cursory inspection, and because of which a thorough reinvestigation becomes necessary. Several ancillary problems are thereby involved, such as preparative procedures, the possibility of more than two chlorophyll components, and the removal of impurities and degradation products. Because the preparation of chlorophyll has been fraught with much uncertainty in the past, we offer a brief description of the method employed. Although no new principle is involved, the procedure is so simplified that we believe the uncertainties to have been largely removed.

EXPERIMENTAL

Preparation of Pigment Extracts

The leaves used in these experiments were maize, wild oats (*Avena fatua*), spinach, alfalfa, and *Heracleum*. Freshly picked

* Carried out under the auspices of the Division of Plant Biology, Carnegie Institution of Washington.

leaves were dried in an air current below 50°, and the dried leaf material was then passed through a Wiley mill. Dicotyledonous plants must be reduced to a meal in a pebble mill. This is not necessary for the monocots, owing to structural differences.

The ground leaf meal is made into a paste with 80 per cent acetone and allowed to steep for 5 minutes. It is then poured into a Buchner funnel and filtered with a slight reduction in pressure, about 5 cm. The deep green solution is then slowly displaced with fresh 80 per cent acetone, about equal in volume to that originally applied. 0.5 kilo of leaf meal may thus be reduced to a straw-yellow or light brown with a total volume of 2 to 3 liters. To the extract is added 0.5 to 0.75 liter of petroleum ether, b.p. 30–70°. The aqueous phase which separates is yellow with grasses, but usually greenish with sunflowers and spinach, owing to some hydrolysis. To conserve the chlorophyll *b*, no serious attempt is made (by washing with aqueous methanol) to eliminate the chief xanthophyll component lutein at this point. When this step was inserted, the yield of chlorophyll *b* was markedly reduced.

The petroleum ether solution is then scrubbed rigorously as follows, to remove the acetone. It is allowed to flow by gravity from a separatory funnel through a long narrow tube (*ca.* 0.5 cm. in diameter[1]), and gently forced 5 to 10 cm. below the surface of a water layer in a second larger funnel directly underneath. This is accomplished by submerging the lower tip of the narrow tube to the desired depth in this water layer.

The petroleum ether is thus scrubbed as it rises to the surface in a fine jet. The jet is controlled by regulation of the flow through the stop-cock of the upper funnel, and the danger of emulsions is eliminated. By passage through 3 to 4 liters of water in a 5 liter separatory funnel, the volume is reduced from 2 or more liters, to about 0.75 liter in two to three washings, owing to removal of the acetone. Five such steps usually suffice for over 90 per cent precipitation of the chlorophyll of large though irregular particle size. With this is occluded possibly half the xanthophyll present, and some waxy material, depending upon the leaf source.

[1] It must encompass the tip of the funnel so that the petroleum ether does not come in contact with the rubber connection.

Preparations involving 1 to 10 gm. of chlorophyll may thus be precipitated in 1 to 2 hours. This crude chlorophyll could be further purified by two to three reprecipitations from ether-petroleum ether, but this was not profitable for our purposes. The crude chlorophyll precipitate in the petroleum ether layer was then centrifuged in 250 ml. Pyrex tubes, and the supernatant liquid decanted. Under the crust of the pigment sediment, there was generally 1 to 5 ml. of water, so the crust was pierced and the water also poured out. The tubes with the sediment were then dried over P_2O_5 *in vacuo*.

Yields varied from about 10 gm. in the case of *Heracleum* to about 2 gm. for maize per kilo of leaf meal. The *Heracleum* and spinach preparations were less friable, owing to the presence of some waxes. A much purer product more closely approximating the crude chlorophyll of Willstätter and Stoll (6) would have been obtained by a talc filtration of the precipitated chlorophyll, followed by a brief wash with petroleum ether. This would have eliminated all the carotene, hentriacontane and similar waxes, and a large portion of the xanthophylls. We wished, however, in at least one preparation, to have introduced no filter aid or adsorbent which contained ash, and talc in fine suspension is not easily removed. In point of fact, particularly with the monocots, the preparations were quite friable when dry, and had very little carotene, as subsequent adsorption proved.

Separation and Isolation of Components

Two variations in Tswett column procedure were tried, with the material precipitated as above. (1) Inulin carefully dried over P_2O_5 was used exclusively for both chlorophyll *a* and *b* preparations. (2) Magnesium citrate was used for removing the bulk of chlorophyll *a*, and chlorophyll *b* was eluted for readsorption on inulin.

With the magnesium citrate ($6H_2O$) the column was cut mechanically, sometimes to remove exclusively the blue zone of chlorophyll *a*, and sometimes to include the light green zone succeeding it, which we shall presently show to contain lutein. In this event, the eluate, transferred to petroleum ether, was washed with small quantities of 85 per cent methanol until the upper layer was pure blue. The hypophasic layer on rapid

saponification gave a bright yellow ether extract (absorption maxima 4750 and 4460 Å., in ether).

An example is given of the second variation in procedure. The citrate is used merely to conserve the supply of inulin. The steps are the same in both cases.

0.5 gm. of crude chlorophyll precipitate from alfalfa was dissolved in 75 ml. of ether, and diluted with petroleum ether until the ether concentration was 20 per cent by volume. This solvent, used by Zscheile (2), was found most satisfactory in increasing the speed of the separation. The solution was adsorbed on magnesium citrate, $6H_2O$, previously stored over $CaCl_2$ *in vacuo*. Column dimensions were 5 cm. diameter by 30 cm. length. When completely adsorbed, the chlorophylls were on the upper third of the column. A small dark zone of pheophytin preceded this (*ca.* 12 cm. from the surface) and a narrow zone of carotene was washed ahead without adsorption. A deep blue zone, about 3.5 to 4 cm. wide, preceded a light green zone, about 4 cm. in width (Zscheile's component *c* to be discussed later), and more strongly adsorbed was a dark green zone of 1.5 cm. width. Above this was a light nondescript zone of greenish gray. All zones were contiguous except for the carotene. After the column had been washed with a total of 0.5 liter of fresh solvent, the pheophytin was separated from the blue by a colorless zone of about 2 cm. The blue and light green zones then occupied about 12 cm. of the length of the column. The total elapsed time was 4½ hours. The column was then cut with a silver spatula, the top 2 cm. being discarded. The dark green zone with a small admixture of its precursor was removed for subsequent purification of chlorophyll *b*, and the second fraction included the remainder of the light green and all the blue zones. Both fractions were covered with acetone and filtered on Buchner funnels, and the pigments completely eluted with fresh solvent.

Isolation of Chlorophyll a—Chlorophyll *a* was not to be readsorbed, and so it was centrifuged to insure removal of particles of citrate. The pigment (in 300 ml. of acetone) was transferred in 100 ml. portions to 75 ml. of petroleum ether, with subsequent addition of water each time to remove the added acetone. The petroleum ether layer was then washed with five portions of 15 ml. of 85 per cent methanol. The lower layers, at first a strong yellowish green, were finally a pale blue-green. The upper layer

in the meantime became an intense pure blue. This layer was then washed with water to remove all acetone and methanol. After the first washing, there is no danger of emulsion formation and the petroleum ether water layers may be shaken mildly. The pigment layer immediately loses its blue color and reddish fluorescence, becoming a rather dark green. (A drop of acetone to the centrifuged mother liquor immediately restores the original appearance.) The layer is drawn off into small centrifuge tubes, centrifuged, and the precipitate in the tube is dried over P_2O_5 *in vacuo*. The yield of dried blue-black flakes was 0.108 gm.

Isolation of Chlorophyll b--The chlorophyll *b* fraction in about 200 ml. of acetone still contained xanthophyll and some chlorophyll *a*. It was transferred to 100 ml. of 20 per cent ether in petroleum ether and the acetone removed with four washings of water. The pigment layer was then dried[2] with 2 to 3 gm. of anhydrous sodium sulfate for 4 to 5 minutes and decanted onto a column of inulin 3 cm. in diameter $\times$ 30 cm. in length. After the material was washed with about 300 ml. of additional solvent, the chlorophyll *b* zone was about 4 cm. in width, about the same distance from the surface, and separated clearly from the blue and light green zones by colorless adsorbent. In some cases in which the proportion of chlorophyll *a* to *b* has not been sufficiently lowered by the initial citrate adsorption, the separation is not so complete and the chlorophyll *b* zone must be eluted and re-adsorbed on a second inulin column. In this case, the chlorophyll *b* was eluted with acetone, and since it was not to be readsorbed, it was centrifuged to eliminate any trace of inulin, precipitated, and dried as in the case of chlorophyll *a*. The time for separation on the adsorbent was about 3½ hours. The yield of dried greenish black powder was 0.048 gm. It should be added that the low recovery was in large part caused by our desire for the highest possible purity in the end-product.

Tests for Purity and Absorption Spectra

The samples listed in Tables I and II were prepared as described. No "best" sample has been selected, so that variation in values indicates only the degree of consistency attained.

[2] This step is a convenience, but not essential. Traces of moisture prevent strong adsorption of the xanthophyll contaminants, and the inulin here need not be quite so carefully dried.

The samples were tested for possible loss of phytol, indicated when pigment is extracted from ether with 22 per cent hydrochloric acid (6). The test was uniformly negative.

Cleavage Test—This presented two problems, the first involving the best method of carrying out the hot saponification of the chlorophyll, the second, a rather precise neutralization of the concentrated alkali. Our best results have been obtained when the chlorophyll, in solution, and the alcoholic hydroxide were heated to boiling, prior to mixing. Under these conditions, with dropwise neutralization with concentrated HCl, the pigments, forced into ether, were extracted readily from it with 5 and

TABLE I

Nitrogen and Ash Content of Chlorophyll Preparations

	Source	Nitrogen	Ash	Adsorbents used
		per cent	*per cent*	
Chlorophyll *a*	Maize	6.20		Mg citrate, blue zone alone
	Wild oats	6.52	4 83	Inulin exclusively
	Heracleum	6.27	4.44	Mg citrate, blue and light green zones
	Alfalfa	6 15	4.55	" "
	Theoretical	6.26	4.50 (as MgO)	
Chlorophyll *b*	Wild oats	6.00		Inulin exclusively
	Spinach	6.03	4.63	" "
	Heracleum	6.44	4.68	Mg citrate, inulin
	Alfalfa	6.12	4 46	" " "
	Theoretical	6 15	4.43 (as MgO)	

9 per cent acids for chlorin and rhodin respectively. The ether solution was barely tinted in the case of chlorophyll *a*, while with chlorophyll *b*, it was virtually colorless. The allomerized material remaining in the ether certainly did not represent 1 per cent of the total, and probably was formed during the test.

Allomerization is evident when samples fail to give a satisfactory Molisch phase test (7). With alcoholic potassium hydroxide, chlorophyll *a* yields a transitory yellow phase, chlorophyll *b*, a red one, with reversion finally to the original colors. With good samples, the colors are pure. The chlorophyll *b* phase, for example, is reddish brown only if other pigments or impurities are

present. The effect of moisture is to make the phase, particularly with chlorophyll *a*, markedly more ephemeral. However, with

TABLE II

Specific Absorption Coefficients of Chlorophyll Versus Wave-Length (λ)

λ	$\log_{10} k$					Remarks
	Corn	Wild oats	*Heracleum*	Spinach	Alfalfa	
			Chlorophyll *a*			
6700	1.677	1 724	1.672	1.630	1.660	Steep part of curve
6630	1.919	1.961	1.923	1.904	1 913	Near maximum
6400	1 073	1 088	1.066	1 075	1.066	Minimum near 6350 Å.
6300	1 068	1 069	1 050	1 047	1 038	
6150	1.199	1.209	1.196	1.193	1.181	Near second maximum
5900	0.894	0.878	0 863	0 852	0.848	
5800	0 922	0.982	0 910	0 938	0.937	Third maximum
5600	0.663	0 712	0 684	0 663	0.666	
5350	0.588	0 643	0 578	0 548	0 589	Pheophytin maximum
5000	0.340	0 394	0.292	0 334	0 314	Very small "
4700	0.084	0 238*	0 113	0 098	0 183	Near minimum
4300	2.006	2 048	2 036	2 016	2 021	Maximum
4100	1.882	1.921	1 892	1 856	1 884	"
			Chlorophyll *b*			
6600		0.986	1.051	1.024		Steep part of curve
6450		1 755	1 697	1.707	1 700	Near maximum
6300		1 185	1 174	1 168	1.163	
6200		0 928	0 929	0 925	0.952	Minimum
5950		1.061	1.052	1 045	1.045	Maximum
5600		0.825	0 819	0 816	0 820	
5300		0.632	0 630	0 637	0.647	
5100		0.473	0.464	0.479	0.492	Minimum
4700		1.813	1 793	1.801	1 762	Steep slope
4550		2.172	2.170	2.158	2.143	Maximum
4350		1.758	1.778	1.765	1 773	"
4300		1 743	1 769	1 753	1 756	Chlorophyll *a* maximum

* The minimum in this sample lies between 4750 and 4800 Å., at about 4760 Å. In all the others, it is between 4750 and 4700 Å., at about 4720 Å.

anhydrous alcohol and sodium hydroxide, the yellow phase of the alfalfa chlorophyll *a* was found to persist as a pure yellow

for over 2 minutes. After 3 minutes, it was greenish yellow, and fully 5 minutes were required before the solution began to resemble the blue of the original. The red phase of chlorophyll *b* was not markedly longer, but faded more imperceptibly to reddish brown, brown, and green.

Analytical Data—On eight of the nine preparations, nitrogen determinations and some ash analyses were made by Mr. H. W.

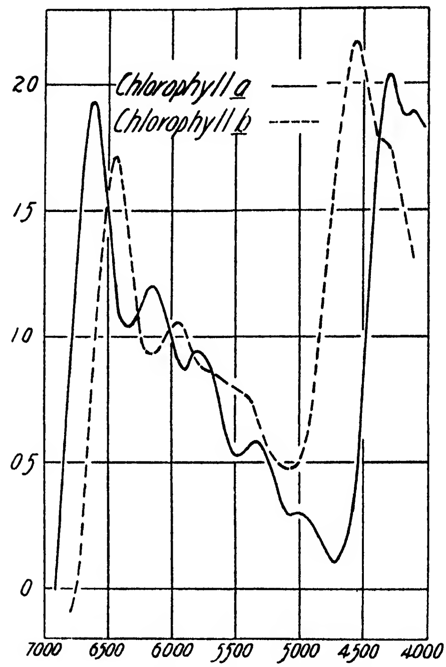

FIG. 1. Absorption coefficients of chlorophyll as a function of wave-length. The ordinate measures the values of $\log_{10} k$; the abscissa, λ in Å.

Milner. These are listed in Table I, where in the last column brief reference is made to the adsorbents used in the preparation.

The analyses were made in accordance with the rigid directions of Stoll and Wiedemann (8).

In the case of wild oats, *Heracleum*, and alfalfa, both components were prepared from single samples of crude chlorophyll.

For the absorption spectra, approximately 2 mg. of a preparation were accurately weighed and dissolved in acetone which had been

dried and redistilled over CaO in an all-glass still. The transmissions were measured at two levels, about 20 mg. per liter and 2 mg. per liter. These concentrations together covered adequately the range 4000 to 7000 Å. over which measurements were made on the spectrophotoelectric equipment constructed by Smith (9). The specific absorption coefficients have been calculated from these measurements. In Fig. 1, the average values of the coefficients are plotted as a function of wave-length, and individual measurements are shown for certain wave-lengths in Table II.

DISCUSSION

Preparation of Pigment Extracts—Plunging the leaves into hot water is necessary to conserve certain of the xanthophyll fractions (10) but is not to be recommended for chlorophyll extraction. In leaves of acid sap, much pheophytin may be formed, and in others, hydrolysis, *i.e.* loss of phytol from the chlorophyll, may occur. This is demonstrated with several species of leaves, *e.g.* sunflower, spinach, and *Heracleum*, at certain seasons, if held in a water bath at 70–80° for 10 to 20 minutes. Solvent extraction of fresh leaves, furthermore, is far from suitable in several cases. The leaves must be dehydrated with acetone before the pigment can be extracted, and aqueous acetone is therefore in contact with the leaf material for a half hour or more before extraction is complete. With leaves rich in chlorophyllase, chlorophyll losses are not inconsiderable.[3] Dehydration of fresh leaves at temperatures of 50° or less is therefore in many instances the safest procedure, as well as the most sparing for solvents.

Isolation of Chlorophyll Components—In the method of Willstätter and Stoll (6), an invaluable step is the precipitation of crude chlorophyll from petroleum ether solution. We have retained this step, which becomes even more crucial in our estimation, in the final preparation of the individual components. Our crude preparations are not so pure as those of Willstätter and Stoll at this point. In our view, separation of chlorophyll *b* and lutein by partition between solvents is not feasible without heavy losses, so we do not attempt to remove xanthophylls by

[3] This topic is under investigation by C. A. Weast of the Division of Fruit Products, and we hope shortly to deal more adequately with this phase.

washing with aqueous methanol. Our crude precipitates were, however, on occasion dissolved in ether and precipitated with petroleum ether, and no difficulty was experienced in this step. Willstätter and Stoll (6) then isolate the two components by partition between solvents. The chlorophyll *a* is obtained by precipitation from a beautiful deep blue solution ((6) p. 164) and the tests applied ((6) p. 165) are convincing evidence of its purity. Their chlorophyll *b* preparation is possibly less satisfactory. Stoll and Wiedemann (11) have replied to the criticism (1, 2) that it may contain 10 per cent chlorophyll *a* as an impurity. Nevertheless the method is time-consuming, and we may anticipate traces of impurities such as the pheophytins or chlorophyllides which would introduce some uncertainty in the nitrogen and ash contents, and even greater error in the more sensitive absorption measurements. The Tswett column therefore becomes inevitable.

Our preference for inulin over sucrose (1) or talc (2) is based on the fact that when most of the chlorophyll *a* has been separated by a preliminary adsorption, the chlorophyll *b* zone containing traces of the other pigments may be readsorbed on a fresh column and obtained as a discrete zone entirely separated from the others. There is therefore no ambiguity as to zones of intermediate composition.

Crude extracts may be used directly on inulin, but owing to its cost, we precipitate the chlorophyll first as already described, removing many impurities, thereby reducing by nearly one-half the quantity of adsorbent required. As an alternative procedure for the preliminary removal of the bulk of chlorophyll *a*, magnesium citrate is used, the inulin being reserved for final purification of chlorophyll *b*. Several adsorbents give results similar to the magnesium citrate, but in common with it, most of them adsorb the chlorophyll *b* zone too strongly for effective separation from the last traces of chlorophyll *a* and other colored impurities. Of these, corn-starch may be the most promising when conditions are carefully standardized.

Chlorophyll c—Very early in these experiments it was noted that carotenoid adsorption was intimately related to the water content of the adsorbent. Magnesium citrate may be obtained containing from 0 to 14 molecules of water of crystallization. Prepared

from citric acid and magnesium carbonate, it contains between 6 and 7 molecules of water. Exposed to air, it readily takes up 1 more H_2O; dried over $CaCl_2$ *in vacuo*, it consistently gives a product of composition $6H_2O$, and over P_2O_5, $5H_2O$. The carotene may in all three cases be washed through a column of magnesium citrate with virtually no adsorption. However, the main xanthophyll component, presumably lutein, behaves very differently. On magnesium citrate with $6H_2O$ or less it is adsorbed rather more strongly than chlorophyll *a*, from petroleum ether solution, and the chromatogram gives three zones, dark green, light green, and blue, identical with Zscheile's illustration ((2) Plate X). If dichloroethane be now added, and the column is of adequate length, the intermediate zone (chlorophyll *c* in Zscheile's nomenclature) is slowly washed through the blue chlorophyll *a*, eventually appearing as a discrete bright yellow band below the blue zone. The width of the chlorophyll *c* zone may be modified at will, by selection of the proper adsorbent. The appearance of the yellow zone in the blue chlorophyll *a* necessarily yields a green color. If we start with magnesium citrate of composition 7 to $8H_2O$, the lower zone appears light green *ab initio*, with a bright yellow fringe at the bottom, and the yellow will separate completely below the blue merely by washing with petroleum ether, without recourse to dichloroethane.

It has long been recognized (12) that for effective adsorption of xanthophylls, the adsorbent must be carefully dried, or the adsorption is but weak. We have here an interesting reversal of position on a column, not between members of the same chemical group, it is true, but between a carotenoid and a chlorophyll, dependent only upon the moisture content of the adsorbent, or upon the solvent employed. The effect is even more striking when the magnesium citrate ($5H_2O$) is employed.

We were unable to obtain as effective a talc preparation as Zscheile (2) used, but it was possible to demonstrate adsorption of carotenoid above the blue zone when a petroleum ether crude leaf extract was adsorbed on Merck's talc dried 12 hours over $CaCl_2$ *in vacuo*. This was shown by saponification of eluted pigments, when an ether extract became bright yellow. In our experience lutein cannot be separated from chlorophyll *b* by partition between solvents. If the alcoholic phase is sufficiently

concentrated to effect a reasonable (90 to 95 per cent) removal of xanthophylls, there are unavoidable and heavy losses of chlorophyll *b*. The fact that Zscheile's 10 month-old preparation of chlorophyll *c* does not behave as a simple mixture of chlorophylls *a* and *b*, whereas the 24 month-old sample does ((2) p. 559), may therefore be ascribed to xanthophyll decomposition on storage. It is not clear why Zscheile, testing for carotenoids, permitted some hours to elapse ((2) p. 549) in a cold saponification of the chlorophyll. It seems the cause of his failure to find carotenoid contaminants. Similar materials may exhibit such large differences in their properties as adsorbents that it would be rash categorically to assert that ours is the explanation of Zscheile's chlorophyll *c*. We have nevertheless observed a phenomenon apparently identical with that described by Zscheile, and our explanation is based on radically different grounds.

Criteria of Purity—On the basis of tests for chlorophyllides, allomerization, and homogeneity on the Tswett column, the respective preparations of the two components were identical, nor can serious exceptions be taken to any of the analytical results which indeed compare favorably with results from other laboratories. In considering next the accuracy of the measurements in Table II, we may briefly consider certain factors. Smith (9) has shown that transmissions are measured accurately within 1 per cent. With slit widths on the monochromators of 0.07 mm., a spectral region of about 13 Å. is isolated in the region 6600 Å. By interpolation, we may estimate the positions of the maxima in this region with an uncertainty of not over ±7 Å. The values of the absorption coefficients (in liters per gm. cm.) have for convenience been grouped into three categories, the maxima, the steep portions of the curve, and the region of low absorption. From Table II, the average values for $\log_{10} k$ for chlorophyll *a* at 6630 and 4300 Å. are 1.925 and 2.025. The greatest single variation here is 0.057 logarithm unit at 6630 Å. for wild oats and spinach. This is an uncertainty of ±7 per cent in the coefficient itself. At 4300 Å. the greatest variation is 0.042 unit or ±5 per cent. The mean deviations of the average for the above wave-lengths are 0.015 and 0.013. These correspond to uncertainties of ±1.8 per cent and less. One may therefore expect the values at these wave-lengths for any individual preparation to deviate by not

more than 3 per cent from the average. Similarly with chlorophyll *b*, the averages at 6450 and 4550 Å. are 1.715 ± 0.01 and 2.161 ± 0.005 respectively. The latter value was remarkably reproducible, and the smaller band at 4350 Å. was scarcely more than a point of inflection in the curve, and the absence of a band near 4300 Å. indicated satisfactory removal of chlorophyll *a*.

The values in regions of rapidly changing absorption show no unreasonable errors. For example, at 6600 Å., the various chlorophyll *b* preparations show a maximum deviation of 0.065 unit, or 16 per cent. From 6700 to 6500 Å., however, the coefficient increases approximately 20 times. An error therefore of ±8 per cent on either side of the average is not significant *as between different preparations*. For individual samples, there is no suggestion of a break in the curve at 6600 Å. Graphically a break of 0.05 logarithm unit would be perceptible at this point, and we may calculate that this would mean about 6.5 per cent chlorophyll *a* in the sample, which cannot be the case, because not only does it fail to show in the chemical tests applied, but also in the absence of increased absorption at 4300 Å.

The minima are less satisfactory in the region 5100 to 4700 Å., particularly in the case of chlorophyll *a*. 1 per cent of carotenoid would have the effect of inert non-colored impurity in the regions of high absorption for chlorophyll *a*, but would significantly modify the value at the minimum, near 4720 Å. Furthermore, to obtain the same accuracy as in the case of the maxima, concentrations of 50 to 100 mg. per liter should be used in this region of high transmission. Unfortunately with a 25- to 50-fold variation in concentration, the uncertainty appears to be doubled. It will require a more exhaustive study to determine whether there are small deviations in Beer's law over this concentration range. The uncertainty at 4700 Å. for chlorophyll *a* is therefore approximately 0.05 unit.

It is *a priori* most likely that the major part of these variations must be ascribed to impurities, and this would seem to be particularly true of the chlorophyll *a* from wild oats. On the basis of its high nitrogen content, a calculation indicates 6 per cent of phytol-free derivative. Assuming that the molar absorption coefficients of chlorophyll and chlorophyllide are equal at 6630 Å., we may calculate about 8.6 per cent of the sample to be

chlorophyllide. This would have been detected in the HCl test. The effect of phytol on the absorption spectrum of chlorophyll has not yet been thoroughly evaluated, as pointed out by Stern and Wenderlein (13), so that in spite of some uncertainty, we have felt it inadvisable to exclude this preparation from the average. To this may be added the point that grasses are ordinarily deficient in chlorophyllase, so that the presence of chlorophyllide is not to be anticipated.

Table III

Comparison of Coefficients of Chlorophyll Components

In benzene	ε, Winterstein and Stein		This laboratory	
			λ, maximum	ε
Chlorophyll *a*	139×10^3		6650	164.5×10^3
	220×10^3		4330	212×10^3
Chlorophyll *b*	110×10^3		6450	123×10^3
	$350\text{–}360 \times 10^3$*		4580	326×10^3
In ether	**Hagenbach *et al.***		**This laboratory**	
	λ	Log ε	λ	Log ε
Chlorophyll *a*	6556 (maximum)	4.859	6600	5.246
	4307 "	5.155	4300	5.350
	4736 (minimum)	3.405	4750	3.420
Chlorophyll *b*	6376 (maximum)	4.857	6425	5 061
	4499 "	5.321	4530	5.498
	5101 (minimum)	3.530	5100	3.769

* This value is given by the authors. The others are computed from their curves ((1) Fig. 1).

We believe therefore that the averages of the coefficients are accurate within ±2 per cent over the greater part of the range studied. In regions of high transmission and where change is rapid, this uncertainty should be approximately doubled. No individual preparation deviates from the average in regions of high absorption by more than about 3 per cent.

Comparisons with Other Laboratories—We have used acetone in preference to ether or benzene, because this solvent is applicable to routine work, and furthermore the other solvents must be used

immediately after elimination of peroxides. For utilitarian reasons, we have reported the specific absorption coefficients k,[4] in Table II and Fig. 1, as $\log_{10} k$, because in this way the characteristic curve for the pigment is demonstrated regardless of concentration, unless of course Beer's law is not obeyed, and, in addition, differences in the absorption in the range of high transmission in the green are more clearly illustrated. For our comparisons, however, with benzene the results are based on ϵ, the molar coefficient with the logarithm to base e, and with ether, results are reported as $\log_{10} \epsilon$, thereby conforming with procedures of other laboratories. Comparisons are shown in Table III.

There can be little doubt that the pigments prepared in this laboratory are essentially similar to those of Winterstein and Stein (1) and Zscheile (2). The ratios of red to blue maxima are, however, higher in our preparations. Thus for chlorophyll *a* we find $(164 \times 10^3)/(212 \times 10^3) = 0.77$; similarly for chlorophyll *b* we obtain 0.38. We calculate Zscheile's values to be 0.76 and 0.35 respectively, and for Winterstein and Stein 0.63 and 0.31. The blue maxima are undoubtedly exceedingly sensitive to traces of xanthophyll, and the red maxima are rendered less sharp and more diffuse, owing to small displacements of the band, if traces of breakdown products are present. We agree with Zscheile in finding no band at 6200 Å. for chlorophyll *b*, but instead a minimum. Our chief divergence with Zscheile is quantitative, our coefficients being higher. This may be due to a more effective removal of colorless impurity, or to spectroscopic technique. The fair gross agreement with Winterstein and Stein is marred in minor details, and in the rather large discrepancy in red and blue maxima ratios already noted.

All three groups are markedly divergent from the preparations described by Hagenbach *et al.* (3). In ether, their chlorophyll *a* maximum is at 6556 Å. (*cf.* Zscheile 6600 Å., Winterstein and Stein 6630 Å., and ourselves 6615 Å. (by interpolation)). A similar discrepancy is to be noted with chlorophyll *b*. Furthermore, in the regions 5500 to 4900 Å. for chlorophyll *a* and 5800 to 5400 Å. for chlorophyll *b*, their curves resemble plateaus with small peaks. With ether, acetone, and also dichloroethane (15) we have found a rapid falling off in absorption, in spite of minor

[4] This is in accordance with Brode (14).

bands. Finally, Hagenbach *et al.* are alone in finding the coefficients for chlorophylls *a* and *b* to be equal at their respective maxima in the red. Whatever, therefore, the initial purity and homogeneity of their preparations, the pigments whose absorption is reported are substantially different from those of the other laboratories.

The results reported by Meyer (4) give emphasis to this view, because he used, in common with Hagenbach *et al.* (3), components purified by Stoll. His curves ((4) Fig. 3, p. 362) are more in keeping with ours, qualitatively, although there are many serious differences. Thus the minimum for his chlorophyll *b* lies at about 5250 Å., for chlorophyll *a* about 5000 Å. (*cf.* ours at 5100 and 4750 Å. respectively). This plays an important part in determining whether solutions are yellow-green and blue-green, or pure green and pure blue, respectively, as is the case with our preparations. The log ϵ values reported by Meyer for chlorophylls *a* and *b* at their respective red maxima are about 4.6 and 4.3 (*cf.* Hagenbach *et al.*, 4.859, 4.857). As between maxima and minima, log ϵ (red maximum) $-$ log ϵ (green minimum) for chlorophylls *a* and *b*, Meyer's differences are about 1.2 and 0.6 respectively (*cf.* ours, 1.846 and 1.292).

Meyer finds a considerable discrepancy between the absorption spectra of the preparations received from Stoll and that of "native" or crude chlorophyll. When the curve for a 9:1 mixture of chlorophylls *a* and *b* is calculated by Meyer and compared with that for the crude material, agreement is good from 6800 to 5600 Å. The former gives rise to a definite band (maximum about 5350 Å.), where log ϵ values are about 0.2 unit higher than for the crude material. Meyer shows that this cannot be accounted for by carotenoids. He is led, in our opinion, to an untenable deduction that "native" chlorophyll differs from "pure" preparations, as generally accepted from the descriptions of Willstätter and Stoll (6). He has noted that the "native" chlorophyll solutions are slowly converted, on standing, into the "pure" chlorophylls, as shown by a study of their absorption spectra. He has not shown that the fault does not lie with his standard "pure" samples, and our confidence in these is severely shaken by his failure to explain the extraordinary differences between his results and

those of Hagenbach *et al.* (3), when both groups worked with Stoll's preparations.

We have been able to duplicate the absorption spectrum of various leaf extracts with "synthetic" mixtures of crude chlorophyll (*i.e.* the chlorophyll *a* and *b* mixture), carotenoids, and in special cases, with admixtures also of pheophytin. This forms a quite important check on our criteria for determining whether there have been significant changes in the isolated components as compared with their condition in the crude extract. This check was carried out with the visual Bausch and Lomb Universal spectrophotometer. The results are not directly comparable with those reported here because of differences in slit widths, etc., but we may find a more direct check with the same photoelectric photometer in the work of Strain ((10) Figs. 20 to 22). Here Curve I, representing total ether-soluble pigments, is qualitatively similar to the curve for Meyer's "native" preparations. Elimination of carotenoid absorption gives rise to Curve II, total chlorophyll, or more appropriately total residual green pigments. The relation of this Curve II to our curve for chlorophyll *a* is what we can predict from the presence of some chlorophyll *b* in Strain's crude extract. However, in spite of the chlorophyll *b* present, Curve II appears to represent a purer chlorophyll *a* *spectroscopically* than does Meyer's standard chlorophyll *a* preparation. This necessarily casts doubt on Meyer's values for his standard.

Spectroscopic Measurement of Chlorophyll Content—The sensitivity of the spectroscopic method to minor changes in the structure of the pigment molecule will be invaluable as a criterion of purity, when certain preparations are accepted as standard. The worker desirous of selecting a standard has four sets of data now, wholly incompatible with each other as a basis for accurate assay, by spectroscopic means. The applicability of the coefficients to this end must form the subject matter of a study in the future. Results must be checked against the chlorin-rhodin method of Willstätter and Stoll, and on a larger scale against chemical methods, such as magnesium analyses. Comparative results by spectroscopic means have much greater significance. The rapid extraction of material from a particular plant with acetone or-

dinarily yields a remarkably reproducible extract for that plant. Differences in absorption at a number of wave-lengths are usually constant for different preparations within the limit of the spectroscopic method. They are then directly related to the concentrations of pigment. If, however, they are not constant, there is the possibility of degradation products or a change in the chlorophyll *a* to chlorophyll *b* ratio (15).

SUMMARY

In this paper we report the values of absorption coefficients for several preparations of chlorophylls *a* and *b*. The coefficients from five different laboratories are so incompatible that their application to spectroscopic assay of the green pigments is useless, until independent verification yields acceptable standards. As an aid to this, and to the correct evaluation of our preparations, we consider methods in some detail, our procedure being considerably simplified. This is particularly true of the step involving the precipitation of chlorophyll from petroleum ether.

As criteria for purity, correct analyses and the various tests, phase, cleavage, etc., are of course essential. When we then apply the highly sensitive spectroscopic method, we find, in the last analysis, that purity is assured only when the pigment is homogeneous and forms an entirely separate zone on the Tswett column. The means to this end depend on the selection and standardization of the adsorbent.

In the foreword to a recent monograph by Dr. Strain (10), Dr. Spoehr has pointed to the closely cooperative nature of the research on the plastid pigments at the Carnegie Institution. It must be obvious to the most casual reader that the author has benefited to the greatest extent from the facilities available and the courtesies and suggestions offered at all times.

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IMMUNOCHEMISTRY OF CATALASE

II. ACTIVITY IN MULTILAYERS

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The use of enzyme proteins as antigens offers an opportunity to investigate the nature of the antigen-antibody union from the point of view of its effect on the enzymic activity. Kirk and Sumner (1) found that the compound urease-antiurease retained its activity, and Campbell and Fourt (2) found a similar activity of the catalase-anticatalase compound. In each case the resuspended precipitate, although active, was less active than the corresponding amount of dissolved enzyme, a decrease attributed to decrease in dispersion. Campbell and Fourt found hardly any loss of activity if the mixture of antigen and antiserum was diluted directly, and a variable loss if the precipitate was resuspended by ordinary shaking and stirring after having been centrifuged down. Although there was evidence against the dissociation of the precipitate, this evidence had to be obtained by extrapolation from the strong solutions in which complete precipitation was obtained to the dilute suspensions in which the activity was measured. The possibility of dissociation in the dilute reaction mixtures could not be ruled out.

An observational check on dissociation is afforded by the use of Langmuir and Blodgett's multilayer techniques (3). Langmuir and Schaefer with Dr. E. F. Porter (4) found that alternate layers would deposit onto a conditioned multilayer plate from solutions of diphtheria toxin and antitoxin, and similar observations have been made for other antigen-antibody pairs (5–8). These depositions show a specificity in general similar to that of the precipitin reaction. Thus, if the treatment with catalase or

antiserum is repeated, the net change of thickness is small compared to the original change; alternation of reagents is required for large gains. Only anticatalase serum gives a large deposit on catalase; normal or non-specific sera give no or comparatively small increments. We have examined the enzymic activity of catalase alternating with anticatalase on such plates. Here the dissociation would be revealed by a loss of thickness and a liberation of enzyme into the solution. These experiments also bear on the problems of the structure of the alternating protein multilayer itself.

EXPERIMENTAL

Method

The technique of preparing the plates and measuring the thickness has been described by others (7, 3). Thorium nitrate was used to prepare the plates for adsorption, and distilled water for washing off excess reagents. The catalase activity is measured by the method of von Euler and Josephson, as specified before (2), the only difference being the omission of 0.9 per cent sodium chloride from the reaction mixture. The chromium-plated strip on which the multilayer was deposited was introduced into 50 ml. of ice-cold reaction mixture, and used like a teaspoon to keep the solution well stirred and itself more free from bubbles. After the plate was removed, further samples were taken to determine the amount of catalase activity liberated into the solution by dissociation or solution off the slide. The thickness of the slide was again measured, and the plate tested with a new reaction mixture. The catalase and anticatalase were prepared as in previous work (2), the anticatalase by Dr. D. H. Campbell.

Results

Alternation of Catalase and Anticatalase—The alternate deposition of catalase and anticatalase, up to five complete pairs of layers, is shown in Fig. 1. The points are grouped to show the frequency of any given value, rather than in sets of two or three as originally obtained from the measurement of successive steps on the same slide. The first layer of catalase produces a median change of 53 Å., and the following anticatalase layer, 56 Å. Thereafter catalase layers of 10 Å. or less net change alternate with anticatalase layers of Δ thickness between 40 and 50 Å.

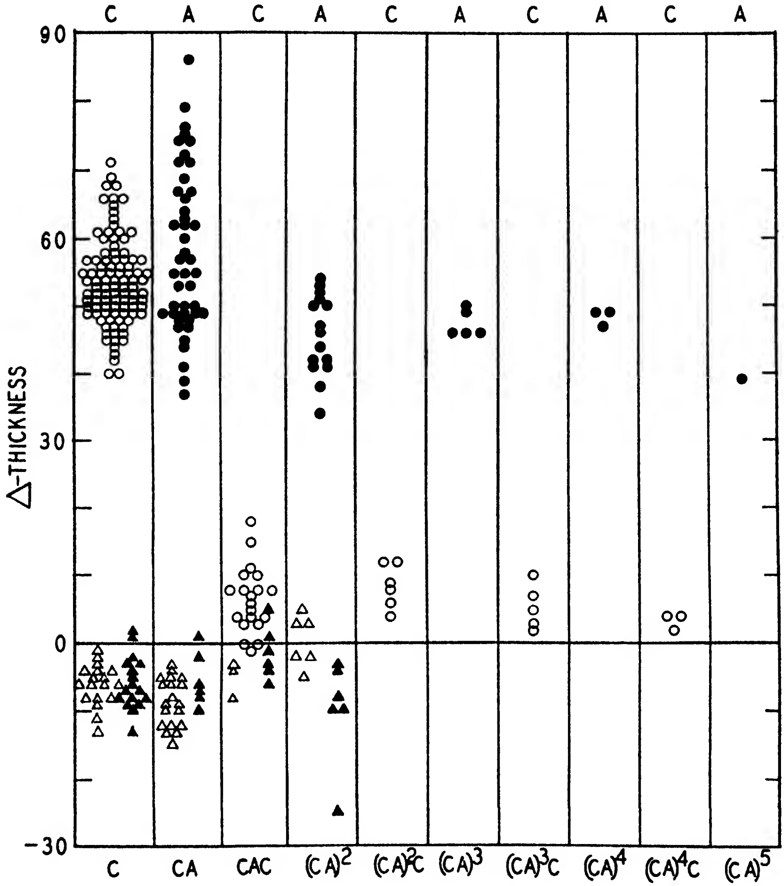

FIG. 1. Thickness measurements of alternating layers of catalase and anticatalase. The ordinate scale gives the change in Å. produced by each treatment. The letters along the top indicate that solutions of catalase (*C*) or anticatalase serum (*A*) were applied to the plate; the structure of the protein multilayer is shown at the bottom. The open circles indicate individual measurements of catalase Δ thickness; the solid circles, of anticatalase. The open triangles mark the change of thickness after reaction with hydrogen peroxide; the solid triangles show the further change with a second period of reaction.

Change of Thickness during Reaction.—The triangles in the lower part of Fig. 1 show the changes in thickness after 7 or 8 minutes immersion in 0.008 M hydrogen peroxide solution buffered at

pH 6.8 by 0.04 M potassium phosphate. The open triangles give the change in the first reaction mixture, the solid ones the change upon retrial. The losses are small and quite uniform in every case, the medians falling between 4 and 9 Å.

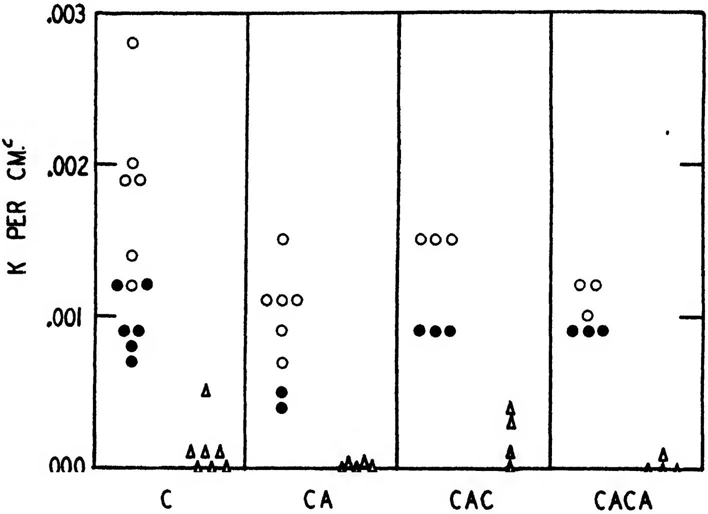

FIG. 2. Enzymic activity of adsorbed catalase and catalase-anticatalase. The ordinate scale represents activity (K per sq. cm.). The different divisions give the activities of multilayers of the structure indicated at the bottom by C for a layer of catalase and A for one of anticatalase. The open circles are for the first trial of activity; the solid circles for another trial in a new lot of reaction mixture. The triangles show the activity left in the reaction mixture after the removal of the multilayer plate.

Activity in Multilayers—The activity per sq. cm. for the different layers of catalase and anticatalase is shown by the circles in Fig. 2. The activity is defined by the equation,

$$K = \frac{1}{t} \log_{10} \frac{a}{b}$$

in which t is the time from the start of the reaction (5 minutes) and a and b are the amounts of potassium permanganate used in titration of a blank and of the sample taken at the time t respectively. The relative uniformity of the activities is the fact most

to be noted; two layers of catalase are no more active than one, and catalase covered by anticatalase is hardly less active than exposed catalase. In every case, the activity found on a second trial (solid circles) is less than that of the first trial (open circles). Samples taken at different periods in a given trial showed rather uniform activity.

Shedding of Catalase—The triangles in Fig. 2 give the activity left behind in solution, per sq. cm. of plate. In every case this is small compared with the activity of the plate, even on its second trial. The nature of the outer layer has a characteristic correlation with the amount of activity which shed; almost no catalase is liberated if the outer layer is anticatalase, but an appreciable amount if it be catalase.

TABLE I

Effect of Reaction on Thicknesses of Subsequent Layers

C indicates a layer of catalase, *A* of anticatalase, and *R* that the layers up to this point were allowed to react with hydrogen peroxide.

Structure of multilayers	Layer in question	Median thickness	Effect of reaction on thickness
		Å.	
C, A	*A*	56	
" *R, A*	"	33	Reduction
" *A, C*	*C*	6	
" " *R, C*	"	15	?
" " *C, A*	*A*	43	
" " " *R, A*	"	25	Reduction
" " *R, C,* "	"	30	"
" " *C, A, C*	C	8	
" " " " *R, C*	"	13	?

Effect on Sandwiching—The alternation of layers continues after a layer has been allowed to react with hydrogen peroxide, but with not as large a Δ thickness. Table I shows that the effect of the reaction is to reduce the thickness of subsequent layers of anticatalase. The changes in the catalase thicknesses are too small to interpret.

Aging—On standing in air overnight, the plates lose the capacity to react with further layers of catalase or anticatalase, and show no activity with hydrogen peroxide.

DISCUSSION

The experiments favor the conclusion that the undissociated catalase-anticatalase compound shows enzymic activity. A necessary qualification is that the union of the two materials on the plates may not be identical with the combination resulting from mixture in solution. Thus, if the reagents are multivalent with respect to each other, as has been proposed by Marrack (9) and Heidelberger and Kendall (10), it may be that the spatial restraints imposed by close packing in a plane may prevent maximal combination. However, the specificity (4–8) shown in the deposition of these layers on the plates may be taken as evidence that the union is of the same type as in the precipitin reaction.

A comparison of the activity of the adsorbed enzyme with the activity in solution is possible. The *Kat. f.* (defined as *K* per gm.),

TABLE II

Activity of Adsorbed Catalase

Layer	Reaction No.	*K* per sq. cm.	*Kat. f.* (or *K* per gm.)	Per cent activity in solution
Catalase	1	0.0019	2600	21
	2	0.0009	1200	10
Catalase-anticatalase	1	0.0011	1500	12
	2	0.0005	700	6

of this particular enzyme preparation, measured in solution, for a reaction period of 5 minutes, was 12,400 ± 400. A *Kat. f.* can be computed for the adsorbed enzyme, by multiplying its volume (area of plate times optically measured thickness) by the density, taken as 1.33. When a layer of catalase alone is compared with a layer of catalase covered by anticatalase, the thickness of the catalase alone is used in each case. The results computed from median values are given in Table II.

This shows that the activity per gm. of adsorbed catalase is only a fifth to a tenth of that in solution. This can only be regarded as a tentative value, for two reasons: (1) The *Kat. f.* in solution is lower than for some solutions of crystalline catalase, which indicates the presence of denatured protein or an impurity which might be preferentially adsorbed, reducing the activity on the plates. (2) The effect of drying and aging may have impaired the activity, independent of the effects of adsorption alone.

We have not attempted to compute a *Kat. f.* for the experiments with more than one layer of catalase, because of the uncertainty as to how much catalase is present. The net change of thickness for catalase layers on anticatalase is around 10 Å., instead of the 53 Å. of the initial layer. The fact that catalase is lost from the plates into the solution when the last treatment of the plates was with catalase and is hardly shed at all when the last treatment was with anticatalase indicates that the layers of material really do alternate. On the simple assumption that there is a second layer of catalase of the same thickness and activity as the first layer the *Kat. f.* would be reduced one-half, since the activity, as shown in Fig. 2, is about the same.

It is rather puzzling that a layer of catalase which can react with hydrogen peroxide, even though it is covered with a 56 Å. layer of anticatalase, does not add its activity to that of the outer layer of catalase. This would suggest that the outer layer might really be an inactive, thin layer, were it not for the evidence from the loss of activity into the solution. The apparent activity of only the outer layer might be explained by a failure of any substrate molecules to penetrate past it to the lower layer.

Langmuir and Schaefer (11) have experimented with catalase layers picked up on multilayer plates after spreading at the air-water interface. The data are not available to compute *Kat. f.* or *K* per sq. cm. values from their work. Similar experiments made in this laboratory have given values of *K* per sq. cm. running through the same range as that found for the layers adsorbed from solution. This is somewhat surprising, since Langmuir and Schaefer have come to doubt that any enzymic activity would be shown by a completely spread protein. The observed activity they attribute to immeshed, unspread molecules. This would lead one to expect a higher activity for layers of molecules adsorbed from solution than for layers of more or less completely spread molecules. The point is one of importance, and cannot be considered settled on the basis of the few experiments so far performed.

Langmuir and Schaefer noted a removal of the catalase from areas of the plate on which bubbles were allowed to form. The losses of thickness which we have noted were uniform over the whole plate. This difference probably has its explanation not only in the difference in modes of deposition, but also in our use

of 0.008 M hydrogen peroxide instead of 3 per cent (0.9 M), and our attempt to dislodge the bubbles as rapidly as possible.

SUMMARY

1. The undissociated compound of catalase and anticatalase, as formed by alternate deposition in Blodgett-Langmuir-Porter multilayers, shows only slightly lower activity than adsorbed catalase alone.

2. The activity per gm. is tentatively estimated at one-fifth to one-tenth of that in solution.

3. The total activity does not increase with two layers of catalase on the plates. This suggests a failure of the substrate to get past the first active layer.

4. The activity of catalase layers picked up after spreading at the air-water interface runs through the same range as that of layers deposited from solution.

5. There is a small loss of material on exposure to the reaction mixture. When the outermost layer is catalase, some enzyme is liberated into the solution, although this activity is small compared with the total. An outer layer of anticatalase permits almost no loss of activity into the solution. This shows that the materials really do alternate in layers in the order of the treatments, even though the measured changes of thickness are different from the thicknesses of the initial layers.

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CHEMISTRY OF THE VAN DEN BERGH REACTION

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The form in which bilirubin exists in the blood is of considerable interest because of the observed differences in the van den Bergh reaction in disease and because of the relation of bilirubin to hemoglobin metabolism.

All of the bilirubin in plasma reacts in 30 minutes with diazonium salts in acid 50 per cent methyl alcohol to give a red dye (1). A variable fraction of the total reacts with varying rapidity in aqueous acid ("direct" reaction). Clinically the size of this fraction is of some diagnostic value in distinguishing obstructive from hemolytic jaundice.

Nearly all of the many theories propounded to explain the differences among plasmas from cases of jaundice in the percentage of the total bilirubin which reacts in aqueous acid can be divided into two classes, (1) those which postulate that the bilirubin not reacting in aqueous acid is prevented from doing so by a definite valence bond to a fraction of the plasma proteins presumably derived from hemoglobin, and (2) those which postulate that catalytic or inhibiting substances present in various amounts produce variations in the per cent of bilirubin giving the direct (aqueous) reaction. Most of the experiments designed to support the latter type of theory have been synthetic, involving the construction of models apparently duplicating, by the use of catalysts, the effects observed in naturally occurring jaundiced plasmas.

The following experiments are an attempt at an analytical solution of the problem by means of the accurate and convenient plasma bilirubin method of Malloy and Evelyn (1). They appear to show, to the exclusion of the second type of theory, that in the plasma from jaundiced patients the bulirubin not reacting in

aqueous acid is attached by a definite valence linkage to a fraction of the plasma proteins.

Cataphoresis—The following experiments show quantitatively that all of the bilirubin is attached to the plasma albumin in jaundiced plasma and moves with it in an electric field.

It has been shown qualitatively that the plasma bilirubin is attached to the plasma albumin (2, 3). Table I shows the results of quantitative experiments in which 2.00 cc. samples of oxalated human plasma of high bilirubin content are subjected to cataphoresis with varying voltages and distances of migration in the apparatus of Coolidge (4) and fractions analyzed after cataphoresis. The bilirubin reacting in aqueous acid in 2 hours is called "direct" reacting. Globulin was estimated after separation from albumin by precipitation in 2.02 M phosphate buffer, pH 7.0 (5). This separation was shown to be unaltered by increasing the plasma dilution 1:120, as was necessary in this case.

TABLE I

Recovery of Albumin and Bilirubin after Cataphoresis

Sample	Original plasma			Cataphoresis fluid			
	Albumin to globulin ratio	Total bilirubin	Bilirubin giving direct reaction	Total albumin recovered	Albumin to globulin ratio	Total bilirubin recovered	Recovered bilirubin giving direct reaction
		mg. per cent	*per cent*	*per cent*		*per cent*	*per cent*
A	1.01	9.0	76	58	50.0	49	59
"	1.01	9.0	76			56	73
"	1.01	9.0	76	62	1.64	38	64
"	1.01	9.0	76	27	1.52	29.6	50
B	0.87	9.05	22	54	19.0	47	
"	Same cataphoresis as above			20	1.2	15	45
C	1.4	7.71	20	21.5	1.4	26	45
"	1.4	7.71	20	24	2.7	19.5	70
"	1.4	7.71	20	27.4	3.2	21.6	62
"	Same cataphoresis as above			26.2	∞	17.3	58

As can be seen from Table I the recovery of the total bilirubin parallels that of the albumin within the limits of accuracy of the method. The agreement is very satisfactory, when one considers

the lability of plasma bilirubin in the absence of the reducing substances present in normal plasma. One can conclude that practically all the bilirubin is bound to the plasma albumin. This has been confirmed by ultrafiltration of plasma through cellophane membranes. No bilirubin passes through, in agreement with the results of Gregory and Andersen (6) and contrary to earlier observations.

A comparison of the per cent of direct reacting bilirubin in the original plasma with that in the cataphoresed fluid shows both increases and decreases. These are difficult to interpret, as the data are obtained on such small amounts of material that the limit

TABLE II

Rate of Reaction of Mixture of Plasmas Compared with Sum of Rates of Components

Reaction time	Bilirubin reacting in aqueous acid			
	Plasma A	Plasma B	Plasma A + Plasma B	Mixture of Plasmas A and B × 2
min.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
10	1.15	1.18	2.33	2.48
30	1.45	1.46	2.91	2.86
60	1.65	1.62	3.27	3.30
120	2.10	1.68	3.78	3.90

Plasma A contains 8.60 mg. per cent of total bilirubin; 25 per cent direct reacting. Plasma B contains 2.22 mg. per cent of total bilirubin; 76 per cent direct reacting.

of accuracy of the method for direct reacting bilirubin is approached. They indicate, however, that if the ability to react in aqueous solution is conditioned by factors other than bilirubin or plasma albumin, such factors are not completely removed by several hours of cataphoresis in a strong electric field and are therefore strongly bound to the albumin.

Reaction of Mixed Plasmas—That the differences in reactivity of direct and indirect reacting bilirubin are due to structural rather than catalytic causes is shown by the effect of mixing equal volumes of two plasmas having very different proportions of direct reacting bilirubin and treating the mixture with diazotized sulfanilic acid. The results are shown in Table II.

The rate of the reaction of the mixture is equal to the sum of the rate of reaction of the bilirubin in the two plasmas. If either catalyst or inhibitor were present in excess in one of the plasmas (as the "catalytic" type of theory would assume, since the plasmas are quite different in their reactivity), such catalyst or inhibitor would, in the mixture, alter the reactivity of the bilirubin in both plasmas and thus change the reactivity of the mixture from that of the sum of the rates of component plasmas. This is not the case. Hence this experiment is evidence against the "catalytic" type of theory.

The summative effect of the individual constituents is the same even if the mixture stands overnight.

Rôle of Bile Salts—That the bile acids might be involved in the reaction with diazonium salts in aqueous solution has been suggested by several writers (7). The following experiments show that, with the analytical methods available, there is nothing to support this suggestion.

The only method sufficiently sensitive and accurate for an investigation of this theory is that of Josephson (8), which gives the sum of free and conjugated cholic acid. This method was tested by analyzing samples of plasma to which known amounts of cholic acid had been added and consistently gave an accuracy of better than 20 per cent. Table III shows that there is no correlation between this sum and the character of the van den Bergh reaction.

Extraction with Organic Solvents—That organic liquids capable of dissolving bilirubin extract the direct reacting bilirubin alone from the plasma has been known in a qualitative way for some time. Table IV shows quantitatively the effect of extracting plasma with butyl alcohol. In these experiments 1 cc. of plasma was run into about 50 cc. of butyl alcohol, containing 1.0 cc. of 0.18 N HCl, with stirring. The precipitate was washed twice with methyl alcohol and any remaining alcohol removed under reduced pressure. The precipitate was taken up in 20 cc. of water and the total bilirubin determined.

The amount of bilirubin extracted by alcohol appears to be about equal to that which reacts with diazotized sulfanilic acid in aqueous acid in the first 10 minutes. The simplest explanation of the data appears to be that the bilirubin reacting in the first

10 minutes is held to the albumin in a complex from which it can be separated by alcohol, that the remainder of the bilirubin is

TABLE III

Relationship between Character of van den Bergh Reaction and Plasma Cholic Acid Level

Diagnosis	Albumin	Globulin	Bilirubin	Plasma cholic acid	Van den Bergh reaction (qualitative)
	per cent	*per cent*	*mg. per cent*	*mg. per cent*	
Leucemia, obstructive jaundice	3.4	3.2	8.08	4.8	Direct
Hepatitis	3 3	2.7	10.3	12.9	"
Subsiding obstructive jaundice.	2.8	4.1	0.86	6.8	Indirect
Biliary cirrhosis (autopsy)	1.2	4 7	2.07	4.5	Direct
Pernicious anemia			1.6	8.3	Indirect
Icterus neonatorum	3.6	1 7	5.7	9.1	"
Normal infant			7.0	10.0	"
Cirrhosis with ascites			0.95	2.7	"
Catarrhal jaundice			13.5	4.7	Direct
Septicemia, 2 wks. jaundice	2.9	3.8	2.3	6.8	"
Sickle-cell anemia	3 9	3.66	1.33	5.0	Indirect
Secondary "	4.45	2.93	1.3	5.0	"
Hyperemesis gravidarum	2.46	2.04	0.71	5.0	Direct
Familial congenital jaundice	4.8	2.3	1.9	3.0	Indirect
Congenital heart failure, syphilis	2.25	3.10	1.4	3.5	"

The van den Bergh reactions in this table were carried out by the method of McNee (9).

TABLE IV

Amount of Bilirubin Reacting in Various Times Compared with Amount Extracted by Butyl Alcohol (Mg. Per Cent)

Plasma No.	Bilirubin				
	Total	Reacting in			Extracted by butyl alcohol
		10 min.	30 min.	120 min.	
1	16.2	8.39	9.77	10.0	8.7
2	15.8	9.43	10.7	11.8	9.3
3	11.82	6.97	7.17	7.31	7.7

attached to the albumin by a comparatively strong valence bond, and that this fraction of the bilirubin reacts with diazonium salts

at a variable, slow rate determined by unknown factors. This explanation is in accord with the observed shapes of the curves expressing the rates of development of color in the van den Bergh reaction in clinical cases. In these there is usually a fairly rapid development of color in the first 10 minutes, followed by a slower change at a rate varying in different plasmas.

Rôle of Alcohol—That the rôle of the 50 per cent methyl alcohol in causing the reaction of the total bilirubin is an entirely reversible one appears in Table V which shows the effect of adding excess methyl alcohol and subsequently removing it by evaporation *in vacuo* below 0°. The course of the reaction before and after this treatment is the same. In these experiments 0.8 cc. of plasma was run into 16 cc. of methyl alcohol, chilled in a dry ice-toluene

TABLE V

Effect of Addition and Subsequent Removal of Methyl Alcohol on Rate of Reaction in Aqueous Solution

Time	Per cent of total bilirubin reacting in	
	Original plasma	Methyl alcohol-treated plasma
min.		
10	61	60
30	65	64
60	68.2	65.4
120	70.0	66.5

bath, 4 cc. of water were added, and the chilled mixture evaporated at about 0° to about 3 cc. It was then made up to a volume of 16.00 cc. on aliquots of which the van den Bergh reaction was run. Both ultrafiltration and cataphoresis indicate that in mixtures of equal volumes of plasma and methyl alcohol, such as are used in determining the total bilirubin, there is no separation of the bilirubin from the albumin. From these facts it appears that the rôle of the alcohol in enabling all the bilirubin to react can be looked on as a purely catalytic one.

Fractionation of Plasma Proteins and Determination of Bilirubin Distribution—If, as appears above, the indirect reacting bilirubin is firmly bound to a fraction of the plasma albumin, it should, theoretically, be possible to fractionate the latter and obtain a protein fraction containing all the indirect reacting bilirubin.

An attempt has been made to fractionate the plasma albumin and to follow the van den Bergh reaction of the fractions. This is rendered extremely difficult by the fact that plasma bilirubin, especially the direct reacting fraction, is sensitive to oxidation and to sunlight, especially in acid solution.

Two sheep and a dog underwent ligation of the common duct and were killed when jaundice developed. Because of the rapid oxidation of the bilirubin the only conclusions that can be drawn from the experiments on the sheep are that much of the bilirubin is associated with the proteins precipitated by ammonium sulfate, pH 6.8, between 61.0 and 72.5 per cent saturation at 30°. The green color of the biliverdin which eventually appears in plasma albumin fractions precipitated three times with ammonium sulfate by the method of McMeekin (10) is confined to the above

TABLE VI

Distribution of Total Bilirubin in Ammonium Sulfate-Precipitated Plasma Protein Fractions (Mg. Per Cent)

Original plasma	Fractions precipitated by ammonium sulfate at saturations of		
	50 per cent and below	50–62.5 per cent	Above 62.5 per cent
2.69 (77.3% direct reacting)	0.08	0.07	0.34

fraction except for a faint blue-green color associated with the fractions separating near the half saturation point.

The experiment on the dog plasma (Table VI) was carried out with a minimum of exposure to light and air and as rapidly as possible, without reprecipitation of the fractions. The order of accuracy is low because of the difficulty in removing the ammonium sulfate from the precipitated proteins. The recovery of total bilirubin is only 12.5 per cent and of indirect reacting bilirubin only 55 per cent. In the dark 200 cc. of the dog plasma were poured into a cylinder containing a mixture of 800 cc. of water and 1000 cc. of saturated ammonium sulfate through which hydrogen was bubbling. After 7 hours the mixture was filtered through asbestos wool. The filtrate was placed in a cylinder containing 800 cc. of saturated ammonium sulfate and nitrogen passed through for 12 hours. The mixture (sp. gr. 1.165) was

filtered. The filtrate received 1.5 liters of saturated ammonium sulfate, and nitrogen was bubbled through it for 12 hours. It was filtered, and the clear, colorless filtrate discarded. The three precipitates obtained in the above filtrations were washed with 4.05 M phosphate buffer, pH 7.0, and dissolved in 250 cc. of water each. The solutions gave a precipitate in 50 per cent methyl alcohol and the total bilirubin was determined in about 40 per cent phosphoric acid. The agreement of this method of determination with that carried out in the usual way was within 1 per cent when applied to the original plasma. Reliable determinations of the direct reacting bilirubin were not possible. It can probably be safely assumed that all the bilirubin recovered was originally indirect reacting, as the direct reacting bilirubin is much more susceptible to chemical destruction than the indirect reacting. The results of this experiment confirm those obtained on sheep plasma.

SUMMARY

All of the bilirubin in human plasma of high bilirubin content is bound to the plasma albumin.

The plasma bilirubin which gives a direct reaction in 10 minutes when the procedure of Malloy and Evelyn is used is attached to the plasma albumin as a dissociable complex.

That which does not give a direct reaction is attached to a fraction of the plasma albumin precipitated by ammonium sulfate at pH 6.8 between 61 and 72.5 per cent saturation—probably by a valence bond.

The rôle of the methyl alcohol in causing all the bilirubin to react is purely catalytic.

The character of the van den Bergh reaction cannot be correlated with the concentration of cholic acid in the plasma.

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THE SOLUBILITY OF PANCREATIC AMYLASE IN SOME ORGANIC SOLVENTS

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The solvents most frequently used for the extraction of amylase from the pancreas have been aqueous alcohol (1–4) and glycerol (2, 5). It seemed advisable to investigate the solubility of pancreatic amylase in a number of organic solvents, since the purification of the enzyme mixture could be considerably simplified through the use of a solvent more selective than the few now in use.

The solvents used in this investigation for the extraction of the commercial amylopsin were the purest which could be obtained and were redistilled for further purification.

The amylase preparations used in the solubility determinations were samples of commercial amylopsin prepared from fresh pancreas. Several lots of the enzyme were well mixed and rebottled. The amylase activity was determined as described elsewhere and was found to correspond to a maltose formation of 170 mg. per mg. of enzyme preparation.

To make the extractions, 1 gm. of the commercial amylopsin was allowed to stand for 1 hour, with occasional shaking, in contact with 10 cc. of the solvent. The residue was washed three times with absolute alcohol and then dried in the vacuum desiccator. The dissolved material could not be recovered by evaporation because so many of the solvents were of high boiling point. The method of Sherman was used for precipitating the amylase preparations. This procedure consisted of pouring the extraction mixture into 7 times its volume of absolute alcohol, and in separating the precipitate. Since absolute alcohol was used in washing all the preparations, a 1 gm. sample of the com-

mercial amylopsin was treated similarly with absolute alcohol to give a preparation to which the others could be referred for comparison. All the preparations were kept in a desiccator over concentrated sulfuric acid until they were used in the determination of amylolytic activity.

Saccharogenic power was used as the basis of amylolytic activity. In the method of Sherman and Thomas (6) this power is determined from the amount of reducing sugars formed from a starch digestion mixture. Instead of the Munson-Walker method for determining reducing sugars, a modification of the colorimetric method of Folin and Wu (7) was used after a comparison of the two methods on identical starch digestion mixtures had shown agreement within the limits of error of the determinations.

The method of determining activities as finally used is described below. Triple distilled water was used for all the preparations. All pieces of glassware were Pyrex and were kept filled with distilled water before use. The starch used was a sample of Baker's C.P. soluble starch. When carefully made up, solutions of this starch were clear and slightly opalescent, with no settling out on standing.

14 gm. of soluble starch were weighed out in a tared 50 cc. beaker. About 25 cc. of triple distilled water were added, and the starch was stirred to a paste which was poured with quick stirring into 400 cc. of boiling water. The solution was kept at the boiling point of water for several minutes and then cooled in running water. 10 cc. of 0.7 M phosphate buffer solution (2 volumes of 0.7 M Na_2HPO_4 plus 1 volume of 0.7 M KH_2PO_4) and 10 cc. of 1.4 M NaCl solution were then added and the solution diluted to 700 cc. The resulting solution contained 2 per cent starch, 0.01 M phosphate, and 0.02 M NaCl. Six 100 cc. graduated cylinders were filled with this solution and placed in a rack in a constant temperature water bath. The remainder of the solution was used to determine the pH.

While the cylinders were being warmed in the water bath, a 100 mg. sample of enzyme preparation was weighed out and dissolved in 100 cc. of triple distilled water of a temperature less than 10°. Since in some instances not all the enzyme preparation

would dissolve, the mixtures were centrifuged to give clear solutions. By use of a calibrated pipette, portions of enzyme solution of 0.2, 0.4, 0.6, 0.8, and 1.0 cc. were measured into five 200 cc. Erlenmeyer flasks. At the same time two 2 cc. portions were

TABLE I

Summary of Amylase Solubilities

The results are expressed in terms of mg. of maltose formed per mg. of enzyme preparation.

Solvent	Fraction not dissolving	Activity of material remaining undissolved	Activity of material dissolved by solvent
Absolute alcohol blank	1.00	208	*
Bis(β-hydroxyethyl) ether	0.90	112	337
Bis(β-chloroethyl) "	0.95	178	†
β-Hydroxyethyl ethyl ether	0.98	169	†
Ethylene glycol	0.97	110	248
Ethyl acetate	0.92	196	†
n-Butyl acetate	1.00	198	*
Isopropyl ether	0.98	149	437‡
n-Propyl alcohol	0.98	200	†
Isopropyl "	0.82	183	8
n-Butyl alcohol	1.00	217	*
Isobutyl "	1.00	186	*
Benzyl "	0.96	188	139
Heptane	0.92	192	†
Benzylamine	0.98	228	245
n-Amylamine	0.36	516	23
Dimethylaniline	0.96	200	†
Saturated aqueous acetamide solution, Sample I	0.23	322	1071
Same, Sample II			1140

* No material dissolved.

† Insufficient quantity recovered for the activity determination.

‡ The amount recovered was so small that only one activity determination could be made.

measured into tared watch-glasses for evaporation at 90° to determine the concentration of the enzyme solution.

The contents of the cylinders were then poured into the respective flasks at intervals of 1 minute. The sixth portion of

starch solution, to be run as a blank, was poured into a flask containing no enzyme solution. As soon as filled, the flasks were placed in a rack in the water bath and were shaken at the end of 10 and 20 minutes. At the end of 30 minutes each flask was shaken, and 5 cc. portions were removed at 1 minute intervals, so as to allow a 30 minute digestion for each. Each of the tubes, containing 10 cc. of water, was heated in a boiling water bath, so that when the 5 cc. of starch mixture were added the mixture would be heated immediately to a high enough temperature to stop the action of the enzyme. The activity of the enzyme was then completely destroyed by further heating in the water bath for 10 minutes.

The tubes were then cooled and filled with water to the 50 cc. mark. From these diluted solutions 1 or 2 cc. samples were taken for colorimetric comparison with a standard maltose solution. Such a comparison would give the number of mg. of maltose formed from 100 cc. of 2 per cent starch solution, and this value divided by the volume of enzyme solution gives the number of mg. of maltose formed per mg. of enzyme preparation.

In this manner, the activities of all the amylase preparations were determined. The results of the investigation are summarized in Table I, which gives the activity of the residues and dissolved material when samples of amylopsin were treated with different solvents. The values given are the averages of five or more activities determined for varying amounts of enzyme solution.

SUMMARY

The amylolytic activity of materials recovered after treatment of commercial amylopsin with a number of organic solvents has been determined.

Ordinary alcohols, esters, and ethers when used as solvents do not yield more active amylase preparations.

Ethylene glycol and its ether yield slightly more active material.

Of all the solvents tried, aqueous acetamide is most selective.

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OBSERVATIONS ON THE RELATION OF UREA AND GLYCINE TO CREATINE SYNTHESIS

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Beard and his collaborators (3–10, 13, 27) have made an extensive investigation into the mechanics of creatine synthesis in the rat, in which the effects of the administration of a variety of substances on (*a*) the creatine concentration in a sample of muscle, and (*b*) the rate of creatine excretion have been studied. Beard and Barnes (3) have concluded that "all amino acids of the protein molecule studied have the power of increasing the normal creatine content of young rat muscle." This view has been developed by Beard and Pizzolato (9, 10) who propound the scheme

Amino acids → glycine + urea → glycocyamine + (CH_3) → creatine

This scheme is important to us, since our demonstration of creatine formation from arginine in the isolated rabbit heart (24) might be a special instance of such a scheme rather than the result of the series of chemical changes we suggested in a later paper (19). Scheme 1 illustrates this possibility. We have therefore examined Beard's evidence carefully, and we conclude that while none of it is inconsistent with the conclusions to which he comes there is nowhere in it proof of their validity. Beard relies on increases in muscle creatine concentration and increases in rate of creatine excretion as indices of creatine synthesis. We contend that neither of these effects of itself provides evidence of creatine synthesis. We shall consider the effects separately.

Muscle Creatine—The creatine content of rat muscle is about 400 mg. per 100 gm., but the creatine concentration in plasma

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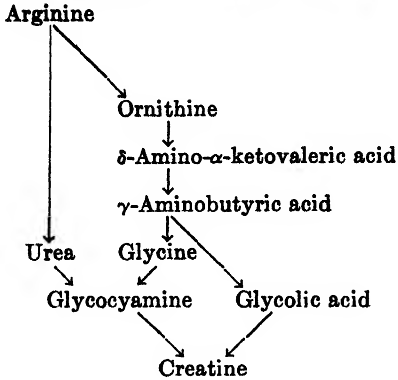
Scheme 1
Conversion of Arginine to Creatine



is about 4 mg. per 100 ml. Tiegs (40) and Eggleton (22) have shown that very little creatine diffuses out of resting frog muscle, while much more diffuses from fatigued frog muscle, and it is indicated that phosphocreatine cannot diffuse across the muscle membrane. Thus most of the creatine, like the major part of the muscle solids, is held inside the cells. Consequently dehydration will increase the creatine concentration without affecting significantly the absolute content in total solids of a particular muscle or the ratio of creatine to total solids. The only safe basis for establishing increases in creatine content of muscles is to refer the creatine content to the total solids or the dry weight of the muscle and to avoid procedures likely to produce dehydration.

In connection with the synthesis of uric acid in birds (23) and with the synthesis of creatine (24) it was emphasised that an established increase in the amount of a substance in an organ or tissue after exposure to a chemical agent is not evidence that the agent has participated in the synthesis of that substance. An apposite instance of this is the demonstration (1, 31, 38) that adrenalin can bring about increases in muscle creatine content (without increasing the percentage of total solids). Mayasama and Riesser (31) found a mean increase of 66 mg. of creatine per 100 gm. of muscle in a series of 2 kilo rabbits into each of which about 2 mg. of adrenalin were injected. Since the muscles constitute 49 per cent of the body weight (Weiske (41)), this corre-

sponds to a total increase in muscle creatine of about 660 mg., more than 300 times the weight of adrenalin injected. Kelly and Beard (27) found that 0.3 mg. of mecholyl causes an increase of more than 100 mg. of creatine per 100 gm. of muscle in 200 gm. rats. If the muscle is considered as 44 per cent of the body weight (Donaldson (20)), this rise in creatine corresponds to an increase of more than 88 mg. in the muscle of the whole animal, more than 264 times the weight of mecholyl used. This drug cannot have supplied 0.5 hydrogen atom per molecule of extra creatine; yet it also produced a striking creatinuria. Beard and Pizzolato (10) injected 0.015 to 0.06 mg. of prostigmine per 100 gm. into rats and obtained 40 to 100 per cent increases in muscle creatine concentration. Even if the highest dose gave the lowest response, this corresponds to an increase in total muscle creatine of 74 mg. produced by the injection of 0.06 mg. of prostigmine, or 0.1 hydrogen atom from prostigmine per molecule of extra creatine. Kelly and Beard (27) appear to feel that there is some difficulty in regarding instances of this nature as syntheses involving the agent producing the effects, since they say, "Under other conditions, such as injection of different purines, prostigmin, etc., the methyl groups of these compounds 'stimulate' this methylation process in creatine formation."

If dehydration is avoided, an established increase in muscle creatine following the administration of test substances may be due to creatine synthesis, but the examples cited indicate that in many instances creatine synthesis, if it does occur, cannot involve the incorporation of any part of the agent provoking the synthesis. *That is, an increase in muscle creatine concentration is not in itself an indication that the agent producing the increase is a source of the extra creatine.* The chemical plausibility of the transformation is irrelevant and treacherous, as the following quotation from Beard and Pizzolato (10) shows. "Prostigmin is the dimethylcarbamic ester of 3-hydroxy-trimethyl-ammonium methyl-sulfate, and, therefore, contains six methyl groups in its molecule. It is very likely that some or all of these were available for the methylation of glycocyamine with resulting creatine formation." It has been indicated that none of the prostigmine molecule can have been incorporated in the creatine whose formation it appeared to stimulate.

In all but the simplest instances, such as the formation of cocarboxylase from aneurin (34), synthesis can be established in an organism or organ only if it can be shown (*a*) that the total content of product in the system (organism plus excreta, or organ plus medium) has increased as the result of the experimental procedure, and (*b*) that the amount of precursor disappearing which is not accounted for by other transformations corresponds to the amount of extra substance formed. Such criteria can be satisfied (24, 19), but they are not satisfied in Beard's experiments. His results are open to an alternative explanation. The available evidence (11, 17, 18, 21, 42, 43) indicates that the rates of absorption of amino acids in the rat, over periods of 3 hours or more, are from 16 to 84 mg. per 100 gm. of rat per hour. Beard and Barnes (3) fed "up to 1 gm." of amino acids to rats weighing 40 to 50 gm. Most of the amino acid must have remained in hypertonic solution in the gut for a long time, and withdrawal of water from the bodies of these small rats may have occurred. The dry weight of the muscle samples was not determined, so there is no check on this possibility. Dehydration may have occurred in later work (5) in which 100 to 200 mg. of amino acid were injected intraperitoneally into rats weighing 60 to 250 gm., since the large amounts of urea to be expected from the catabolism of the amino acids (28) will require the excretion of extra water, and care must be taken to provide an adequate water supply for the experimental animals.

Kayser (26) and Rose (39) suggested that Beard's doses of amino acids are so high that toxic effects might be expected. Absence of deaths or of abnormal behavior does not answer this objection. The injection into rabbits of amino acids in amounts of 3 gm. per kilo of body weight may elicit sympathicomimetic responses (2, 30) which are abolished after adrenalectomy (2), a point of importance in connection with the effect of adrenalin on muscle creatine (1, 31, 38). Lewis and Luck (29) found that the injection of glycine into rats in amounts corresponding to those used by Beard has a depressant action on metabolism and, as the dose is raised, a frankly toxic effect. Many amino acids exhibit a large specific dynamic action. Beard and Barnes (3) regarded the specific dynamic action as irrelevant, since there was no close relation between it and the magnitude of the effect of

the amino acid on muscle creatine. But one cannot ignore the specific dynamic action if there is a possibility that the muscle creatine concentration is being affected by a dehydration dependent on (*a*) the solubility, (*b*) the molecular weight, and (*c*) the rate of absorption of the amino acid in question, since the total effect of the amino acid will be a function of four of its properties which do not bear a simple relation to one another. It is noteworthy that tetrahydro-β-naphthylamine (31, 36) and dinitrophenol (14), which have a stimulant action on metabolism, increase the muscle creatine of rabbits.

Creatine Excretion—Since the difficultly diffusible phosphocreatine in muscle may break down into freely diffusible creatine, it is obvious that a variety of agents may affect the access of muscle creatine to the blood stream. Increased access to the blood stream, however, does not necessarily mean increased creatine excretion. Considerable amounts of creatine may be introduced into the blood stream without any increase in excretion (Hunter (25)), and Pitts (37) has shown that in the dog and man the effect of increasing the plasma creatine concentration on the creatine to inulin clearance ratio indicated active and efficient tubular reabsorption of creatine. The creatine and creatinine contents of rat blood (16) are 4.3 and 1.4 mg. per 100 ml., respectively (both expressed as creatinine). Boggess and Beard (13) report normal daily excretions of creatinine of 1.5 to 3.0 mg., and of creatine of 0.0 to 1.0 mg. These observations suggest that creatine is also actively reabsorbed in the rat kidney, and the blood data indicate that if the tubules were completely inactivated the creatine excretion may be about 3 times as great as the creatinine excretion. Since the maximum creatine to creatinine ratio observed by Boggess and Beard was 0.7, no great tubular impairment would be necessary to produce the creatinurias they have observed. Such creatinurias might be due to diminished efficiency of reabsorption during the urea diuresis to be expected after amino acid injection. This is supported by the observation (8) that the injection of water or physiological saline into rats produced a creatinuria comparable to that observed after injection of amino acids.

It is clear that *increase in rate of creatine excretion is not an indication of increase in rate of creatine synthesis from the agent*

inducing the creatinuria. The fact that creatinuria may be induced by many non-specific procedures—giving selenite (15) or phosphorus (32), for example—supports this proposition.

In a number of instances Beard has shown that an administered substance produces increased creatine excretion and increased creatine content per unit of wet weight of muscle. That this dual response to the same substance does not increase the probability of creatine synthesis having occurred is shown conclusively in work on rats, guinea pigs, rabbits, and hens (16, 35, 33, 32), which indicates that fasting results in a rise in muscle creatine contemporaneous with the creatinuria of fasting known to occur in these species (25).

The outcome of this discussion is that Beard's claim that urea together with glycine may participate in creatine synthesis is not proved. Bodansky (12) failed to obtain any increase in muscle creatine concentration after the administration of glycine to rats. It is claimed (9) that failure was due to the high muscle creatine concentration of Bodansky's control rats and to the short time allowed between glycine injection and death. Since the discrepancy between Bodansky's results and Beard's in the investigation of a key substance in Beard's scheme has made the position obscure, we have performed experiments of two types: (1) repetition of Beard's experiments on the effect of injecting urea and glycine, under his published conditions, but with rats with *lower* muscle creatine concentrations than Beard's; (2) perfusion experiments in which large but "physiological" amounts of urea and glycine were added to the Ringer-Locke solution perfusing an isolated rabbit's heart.

EXPERIMENTAL

Injection Experiments—We have observed the conditions of Beard and Pizzolato (9); namely, injection of 100 mg. each of glycine and urea into rats weighing 100 to 250 gm., and analysis of samples of muscle taken 24 hours later, in which circumstances these authors found in four animals an increase in muscle creatine content of 50 per cent of the amount found in their controls. Our method of determining total creatinine has been described elsewhere (24). Our method of sampling the muscle may have been the source of important differences between these results

and Beard's. Only this reference to the method of sampling was found (3), "... muscle tissue was removed from the hind legs, cut up fine with scissors, and analyzed for creatine..." Since the creatine content of different hind limb muscles in the same species may differ widely (25), it is possible that Beard's method of sampling might be a source of large individual errors. Data on this point for the rat were lacking, so a 200 gm. male rat was taken and four different muscles from each hind limb were analyzed for creatine. (We are indebted to Dr. S. Zuckerman for the dissection of these muscles.) The whole muscle was extracted and determinations were carried out on aliquots of the extract. The results are presented in Table I. The correspondence between similar muscles indicates that analytical error cannot account for the large differences seen between different muscles.

TABLE I

Creatine Content of Rat Hind Leg Muscles

Muscle	Total creatinine, mg. per 100 gm.	
	Right leg	Left leg
Rectus femoris	340	348
Tibialis anterior	355	375
Semimembranosus	443	446
Gastrocnemius	299	305

In the rat, then, different hind limb muscles may also have widely differing creatine contents, and there are potentially large errors in the method of taking "muscle" from the hind limb, mincing, and sampling.

The statistics published by Beard appear to indicate that these errors are not realized in practice. Beard and Pizzolato (9) give a mean value for 132 controls of 420 ± 2 mg. of creatine per 100 gm., indicating a very small variance in their data. But they also state that the mean of all their controls (from the same colony on the same stock diet) for the preceding 10 years was 390 mg. per 100 gm. There is only one chance in about 10,000 that a mean of 390 could be obtained in a sample of data from the same population as that from which the value 420 ± 2 was obtained. There is therefore some obscurity in these published statistics.

In the present experiments the gastrocnemius was dissected from each side in a standard manner, divided transversely into a proximal and a distal half, and the proximal half of one muscle and the distal half of the other taken for total creatinine determination. The remaining halves were taken for dry weight. After the injection of urea and glycine each animal was placed in an individual cage and given free access to food and water. A series of control animals was injected with an amount of the non-metabolized sugar xylose which was isosmotic with the urea and glycine injected into the experimental animals. These controls were intended as indicators of the adequacy of the experimental conditions to obviate dehydration. Normal access to food is

TABLE II

Injection Experiments on Rats

The values are given in mg. of creatine per 100 gm.

Uninjected controls		Injected controls		Urea + glycine	
Wet weight	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight
336	1503	399	1659	368	1562
380	1587	383	1629	387	1635
368	1561	355	1545	344	1523
374	1467	377	1641	367	1608
392	1682	350	1563	355	1589
388	1632	340	1515	316	1484
373 ± 8	1572 ± 33	368 ± 9	1592 ± 24	356 ± 10	1566 ± 23

important, since the injection of glycine into an animal deprived of food might be considered tantamount to an accelerated fast, and might cause a non-specific rise in muscle creatine. It is not clear whether Beard's animals had access to food.

The results of a series of experiments carried out under these conditions are collected in Table II. Although each of the series in Table II is small, the statistics calculated for the creatine content per unit of dry weight show that there is less than one chance in 100 that in another series of controls of the same size the mean value would differ from that observed by more than ±96 mg. per 100 gm. of dry weight, or only one chance in 200 of obtaining a mean exceeding the observed control mean by more than 96

mg. per 100 gm. of dry weight. If in a urea plus glycine group one observed an increase of as much as 6 per cent, this would be a highly significant difference. The methods are adequate to detect effects much smaller than those claimed by Beard and Pizzolato for urea and glycine, but the results allow us to state only that there can be no effect greater than a systematic increase of 6 per cent in muscle creatine. Since the magnitude of increase compatible with these results is entirely incommensurate with that claimed by Beard, we think that the increases he observes are due to the operation of one or more of the sources of error already discussed. If this is correct, then no part of the observed increases need be a specific effect of glycine and urea.

Perfusion Experiments—The conclusions drawn from the injection experiments were tested by perfusion experiments which have already provided unequivocal evidence for the synthesis of creatine in a biological system (24, 19). The technique of the perfusions and determination of total creatinine was precisely as described (19). Nine perfusions of hearts from rabbits weighing more than 2 kilos have been performed, and thirteen further observations were made on hearts taken from control rabbits. Urea (40 mg. per 100 ml.) and glycine (20 mg. per 100 ml.) were present in the perfusate in each experiment. In Table III are presented the observed values for the total creatinine of the heart plus perfusate and the most probable values for the total creatinine of control hearts of the same dry weight as the perfused hearts. The mean difference between the observed value and the value expected in a corresponding control heart is not significantly different from 0. These perfusions therefore provide no evidence for the capacity of the isolated rabbit heart to make creatine from urea and glycine. If the earlier series of perfusions (24, 19) are examined in the same way, with the full series of control animals now available, the data of Table IV are obtained. Addition of arginine, or of glycocyamine plus sodium glycolate, results in a significant increase in the total creatinine of the system heart plus perfusate, while addition of glycocyamine alone, or of glycolate alone, has no effect. The concentrations of the various substances used in these experiments are arginine, 0.6 mM; glycocyamine, 1.0 mM, plus sodium glycolate, 2.0 mM; urea, 6.7 mM, plus glycine, 2.7 mM. It is clear that failure to obtain a positive effect with

TABLE III

Perfusion Experiments on Rabbit Hearts

The "expected" values are calculated from the line of closest fit to 58 control observations which has the equation, $Y = 4.24x + 2.94$, where Y is the total creatinine in mg. and x is the dry weight of the heart in gm. The standard error of the mean difference between the amount found and the amount expected is of such a magnitude that there is one chance in ten of obtaining a mean difference of 0 in a similar series of perfusions. The mean difference is therefore not significantly different from 0.

Dry weight of heart	Amount found	Most probable value in control heart of same dry weight	Difference between amount found and amount expected
gm.	*mg.*	*mg.*	*mg.*
1.45	9.58	9.09	+0.49
0.76	7.27	6.17	+1.10
2.26	13.65	12.52	+1.13
1.02	7.65	6.59	+1.06
1.46	8.06	9.13	−1.07
1.11	9.20	7.65	+1.55
1.68	11.04	10.07	+0.97
1.00	6.12	7.18	−1.06
0.86	7.53	6.59	+0.94
			0.568 ± 0.322

TABLE IV

Summary of Rabbit Heart Perfusion Experiments

Type of perfusion	No. of experiments	Mean difference between observed and expected total creatinine	P*
		mg.	
Arginine	18	+1.245 ± 0.205	0.001
Glycocyamine + sodium glycolate	11	+1.184 ± 0.244	0.01
Glycocyamine	9	−0.654 ± 0.279	0.05
Sodium glycolate	9	−0.541 ± 0.329	0.3
Urea + glycine	9	+0.568 ± 0.322	0.1

* The values of P in the last column express the probability of obtaining a mean difference of 0 in another series of similar perfusions.

urea plus glycine in these conditions indicates that even if there were independent proof that these substances could act jointly as a source of creatine their importance must be small compared with

that of arginine. The conclusion drawn from these experiments is that there is not at present sufficient evidence to require the hypothesis that creatine is formed in muscle tissues from urea and glycine.

SUMMARY

1. Reasons are adduced for thinking that investigations into the effect of administering amino acids and other substances on the creatine content of muscle or on the rate of excretion of creatine cannot provide evidence about creatine precursors in the body.

2. Controlled experiments on the effect of injecting large amounts of urea and glycine on the creatine content of rat muscle have been performed. No increase was observed.

3. Perfusions of isolated rabbit hearts with urea and glycine were carried out in the same conditions in which a quantitative conversion of arginine to creatine has been demonstrated. There was no detectable synthesis of creatine.

4. It is suggested that the available evidence does not require the hypothesis that urea and glycine together are precursors of creatine in muscle tissue.

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THE DETERMINATION OF PURINE NUCLEOTIDES AND NUCLEOSIDES IN BLOOD AND TISSUES

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The method previously described by Kerr and Blish (1932) for the determination of purine nucleotides in trichloroacetic acid extracts of tissues has been simplified. It has also been extended to permit the separate estimation of adenine and hypoxanthine by the method of Hitchings (1933). In the subsequent discussion reference is made to details of technique only where these have been restudied or altered. Further details of the method will be found in the original article (Kerr and Blish, 1932).

Precipitation by Uranium—Ostern and Parnas (1932) recommended that the precipitation by uranyl acetate be carried out at pH 6.4, but we find it to be complete between pH 5 and 8.3, and no precipitation of added nucleoside (guanosine) occurred at pH 5, 6.4, or 8.3. Suitable acidity may be obtained by the addition of 1 drop of 5 per cent acetic acid per 10 cc. of neutralized filtrate. The pH of a 10 per cent trichloroacetic acid filtrate previously neutralized to phenolphthalein is shifted from 8.3 to approximately 6.8 in this way.

The addition of phosphate was previously recommended because of the statement made by Jackson (1923) and confirmed by Buell and Perkins (1928) that nucleotides are precipitated quantitatively by uranium only in the presence of phosphate. When the method was adapted to include estimation of adenine and hypoxanthine, it was found necessary to use larger samples (105 cc. of filtrate), but the quantities of uranyl acetate and phosphate previously recommended resulted in the precipitation of so much uranium phosphate that the subsequent removal of

uranium caused losses of purine by adsorption. In order to avoid this we investigated the possibility of omitting phosphate, and have found that muscle, brain, and blood filtrates contain sufficient phosphate ions to insure complete precipitation of the nucleotide. The amount of uranyl acetate solution required is thus also reduced, 0.7 cc. of an 8 per cent solution being sufficient for samples representing a gm. of resting muscle, and 0.5 cc. for blood filtrates equivalent to 5 cc. of whole blood. When the quantity of inorganic phosphate is increased (as for example in autolyzed tissue), the quantity of reagent should be increased in order to provide a slight excess, which can be noted by the yellow color of the supernatant fluid after the precipitate has settled.

The precipitation of the nucleotides by uranium does not require the half-hour period previously recommended; hence the precipitate may be centrifuged at once.

Hydrolysis of Nucleotide—We have confirmed the statement of Lohmann (1931) and of Levene and Tipson (1937) that the purine is completely liberated from adenine nucleotide within 15 minutes by hydrolysis in N HCl at 100°. If the uranium precipitate is dissolved in 5 cc. of 1.2 N H_2SO_4, the resulting solution becomes approximately normal, and 20 minutes hydrolysis insures complete liberation of the purines.

Precipitation of Purines by Copper Hydroxide—We attempted to eliminate the precipitation by copper hydroxide by carrying out the copper-bisulfite precipitation in the neutralized hydrolysate after removal of uranium. With large samples (3 mg. of purine nitrogen) the method of precipitation recommended by Hitchings (1933) gave good results, but with samples as small as those used in our procedure (1 gm. of muscle, 5 cc. of blood) losses occurred during the centrifuging and washing of the copper-bisulfite purine precipitate. Hence we continue to recommend the precipitation by copper hydroxide when only total purine is to be determined, 1 cc. of 10 per cent copper sulfate being sufficient for the samples used. Peham (1937) also found 1 cc. of 10 per cent $CuSO_4$ to be the minimum quantity required, larger amounts merely increasing the precipitation of non-purine nitrogenous impurities.

When adenine and hypoxanthine are to be determined, the copper hydroxide precipitation of the purines must be omitted,

for in the subsequent removal of copper as cuprous sulfide adsorption of purine is increased when much copper is present (Hitchings, 1933). Since a larger sample (105 cc. of filtrate) is used when the purines are to be separated, direct precipitation of the purines by copper and bisulfite in the uranium-free hydrolysate produces a precipitate of sufficient bulk to permit washing without danger of loss.

If the copper hydroxide precipitate resulting from use of 1 cc. of 10 per cent copper sulfate is dissolved in 1 cc. of N H_2SO_4, the resulting acidity is low enough to permit the quantitative precipitation of purines by bisulfite without previous neutralization.[1] Immediately after addition of the bisulfite to the *hot* solution of the copper sulfate and purine the mixture should be stirred vigorously with a glass rod to insure precipitation of the reddish brown cuprous bisulfite. This when centrifuged forms a more compact mass than the white cuprous-purine-bisulfite complex alone; hence during washing in the centrifuge there is less danger of loss. The few particles of brown cuprous bisulfite which may persist in floating on the surface may be ignored.

Determination of Nitrogen—For ashing we have adopted the digestion mixture of Umbreit and Bond (1936) but have made the water content twice as great as that recommended by these authors so that sodium sulfate may not separate in unheated laboratories.[2] A digestion period of 15 minutes after appearance of SO_3 fumes is ample for purines.

Good results may be secured with the simple distillation apparatus described in Fig. 1, in which the connection to the flask is made with a rubber stopper. The latter serves to hold the flask when the electric heater is lowered at the end of the distillation period. A strong and steady source of heat is essential to insure smooth distillation. We have found 350 watt electric heaters preferable to Bunsen burners. The distillation should be at a rate of about 2.5 cc. per minute, and should be continued for 6 minutes, the last 3 of which serve to wash out the tube.

[1] Edlbacher and Jucker (1936) state that addition of sodium acetate appears to be necessary in spite of the opposite view of Kerr and Blish, but present no evidence to support this statement.

[2] The modified formula is as follows: H_2SO_4 (nitrogen-free) 500 cc., H_2O 1000 cc., $CuSO_4 \cdot 5H_2O$ 2 gm., SeO_2 2 gm., and Na_2SO_4 100 gm.

Each experimenter should redetermine these factors for his own apparatus by distilling known quantities of ammonium salts.

In the titration of excess acid after distillation of the ammonia we have given up the Tashiro indicator previously recommended,

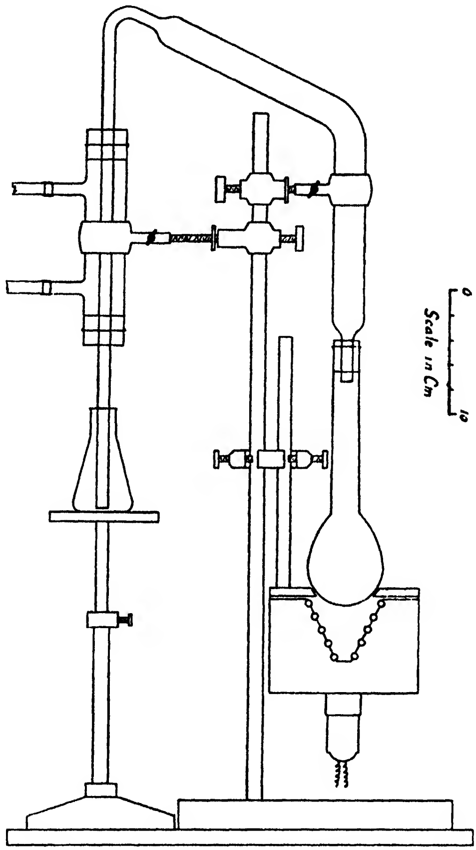

FIG. 1. Distillation apparatus for nitrogen determination by the micro-Kjeldahl procedure.

as methyl red serves equally well. A sharp end-point is obtained only when the 0.01 N NaOH and HCl are prepared from water entirely free from CO_2 and silica.

Revised Micromethod for Total Purine Nucleotide Nitrogen—The protein-free filtrate is prepared as previously described (Kerr and

Blish, 1932). If information regarding the amount of hydrolyzable phosphate attached to the nucleotide is desired, organic phosphate hydrolyzed in N HCl at 100° should be determined (Lohmann, 1928) and this in turn requires that the sum of inorganic and phosphocreatine P be determined without delay (Fiske and Subbarow, 1929, *a*). After a portion of the filtrate is set aside for these determinations (12 cc. for muscle or brain), the remainder may be neutralized to phenolphthalein with 40 per cent NaOH and then barely acidified with 5 per cent acetic acid.[3] Samples containing from 0.2 to 0.6 mg. of nucleotide nitrogen (0.5 to 1.0 gm. of muscle, 1 gm. of brain, 5 cc. of whole blood) are measured in duplicate into centrifuge tubes with conical pointed tips.

Procedure

To each 10 cc. of neutralized tissue filtrate add 1 drop of 5 per cent acetic acid and a slight excess of uranium acetate solution[4] (0.7 cc. per gm. sample of resting muscle, 0.25 cc. per cc. of whole blood) and stir.[5] Allow the precipitate to settle enough to ascertain that the supernatant fluid is slightly yellow, and then centrifuge. Reserve the supernatant fluid for the determination of nucleoside plus free purine.

Dissolve the precipitate[6] in 5 cc. of 1.2 N H_2SO_4 and hydrolyze the nucleotide by immersing the tubes in boiling water for 20 minutes.[7] Neutralize to phenolphthalein with 10 per cent NaOH, render slightly acid with 5 per cent acetic acid, and separate the uranium precipitate by centrifugation. Transfer the supernatant liquid into a 25 cc. centrifuge tube with a pointed conical tip. Instead of washing the precipitate dissolve it in 1 cc. of N H_2SO_4, rinse down the sides of the tube, and reprecipitate by neutralizing

[3] Correction for the change in volume during neutralization must of course be made in calculating the tissue equivalent of the samples.

[4] Prepared by saturation at room temperature (about 8 per cent).

[5] Keep a separate rod for each tube in order to avoid loss of the precipitate which clings to the rod.

[6] When tissues which have been subjected to autolysis are being analyzed, the uranium precipitate should be washed twice with diluted (1:10) uranium acetate reagent acidified with 0.5 cc. of 5 per cent acetic acid per 100 cc.

[7] After hydrolysis of the uranium precipitate the quantity of various reagents need not be varied according to the size of the sample.

as before. Centrifuge and add the supernatant fluid to the tube containing the main portion of the purines.

To the combined purine solutions add 1 cc. of 10 per cent copper sulfate solution and make faintly alkaline to phenolphthalein by means of N NaOH. The precipitate, containing the purine, may be centrifuged at once. Discard the supernatant fluid. Dissolve in 1 cc. of N H_2SO_4 and place the tube in boiling water. When hot add 1 cc. of 40 per cent sodium bisulfite solution and at once rub the inner walls of the tube with the stirring rod to hasten the separation of the reddish brown cuprous bisulfite. After heating for 3 to 5 minutes rinse the stirring rod and the walls of the tube with a fine stream of hot water. Allow the precipitate to settle; then squirt a little hot water on the surface of the liquid to sink any floating particles. Centrifuge sharply for at least 10 minutes, decant, and wash the precipitate once with hot water.[8]

To the washed precipitate add 3 cc. of digestion mixture[2] and wash into a 100 cc. Kjeldahl flask. Add a 6 mm. glass bead, evaporate over a free flame until fumes appear, and then digest over a low flame for 15 minutes. Cool, add 15 cc. of ammonia-free water, and determine the nitrogen content by distillation as previously described (Kerr and Blish, 1932) with methyl red as indicator.[9]

Macromethod for Determining Individual Purines—A sample containing 2 to 3 mg. of purine nitrogen is required. For muscle or brain 105 cc. of neutralized trichloroacetic acid filtrate, equivalent to about 10 gm. of tissue, are a convenient sample. This is measured in portions of 35 cc. each into three 50 cc. centrifuge tubes with conical tips, one of which should be graduated at 15 cc.

[8] A second washing with water often results in a colloidal suspension. To avoid this Schmidt (1933) recommended washing with 1 per cent acetic acid, while Edlbacher and Jucker (1936) found this also ineffective and used 1 per cent acetic acid containing copper sulfate. In our procedure the copper hydroxide precipitate eliminates so much of the non-purine nitrogen that one washing of the bisulfite precipitate is sufficient. It is possible that under some circumstances (*e.g.* autolyzed tissue, or tissue digested with acid) the presence of increased quantities of nitrogenous material may make it advisable to wash three times, in which case the wash liquid recommended by Edlbacher and Jucker should be used.

[9] The methyl red should be recrystallized from alcohol and then dissolved in 50 per cent redistilled alcohol to the point of saturation.

The uranium precipitation is carried out as described for the microprocedure. In order to bring the material all into one tube, dissolve one of the precipitates in 2 cc. of 10 N H_2SO_4, transfer to the second tube, and finally to the third (the one provided with a graduation mark at 15 cc.). The washings from each tube are likewise transferred to the next, with care to keep the final volume within 15 cc. The resulting acidity is approximately normal (0.3 to 0.5 cc. of the acid being required for solution of the precipitate). The nucleotide is next hydrolyzed by immersing the tube for 20 minutes in boiling water, after which the solution is neutralized with 10 per cent NaOH and then made slightly acid with acetic acid. The uranium precipitate is separated, redissolved in sulfuric acid, and reprecipitated as in the micromethod, and the washings combined with the main body of the solution of free purines in a 50 cc. pointed centrifuge tube. The copper hydroxide precipitation is omitted, for reasons already given.

From this point the procedure followed is that of Hitchings (1933) with slight modifications. The following condensation of his method is taken verbatim from the dissertation.[10]

"The centrifuge tube [containing the free purines] was placed in a boiling water bath, and when the solution was hot, 0.8 ml. of saturated sodium bisulfite solution,[11] and 1 ml. of 10 per cent copper sulfate solution were added, and the heating continued for 3 minutes. The precipitate then was centrifuged down, and was washed twice on the centrifuge with 4 ml. portions of hot water. The precipitate was stirred up with 3 ml. of 3 normal hydrochloric acid, and this mixture was heated to boiling over a free flame.[12] The solution was diluted to about 10 ml. with boiling water, and immediately was placed in a boiling water bath, where a stream of hydrogen sulfide was passed through it. When the decomposition was complete (about 3 minutes), the mixture was transferred to a 25 ml. volumetric flask,

[10] We wish to thank Dr. Hitchings for permission to publish this portion of his thesis. Each step of the procedure represents the result of painstaking research which is fully recorded in the complete thesis. Microfilm copies of the complete dissertation (87 pages) may be obtained from the director of the Harvard University Library (Mr. Keyes D. Metcalf), Cambridge, Massachusetts, at a cost of not more than $3.00.

[11] Or 1 cc. of a 40 per cent solution.

[12] A number of lost determinations due to breakage at this point convinces us that heating in a boiling water bath is preferable.

cooled to room temperature and diluted to the mark. After mixing, the solution was poured through a 7 cm. nitrogen-free filter paper."[13]

This solution may now be used for the separate determination of total purine nitrogen by the micro-Kjeldahl procedure, and of adenine and hypoxanthine as described below. We determine total purine nitrogen by ashing 3 cc. aliquots as in the micro-procedure. The Hitchings procedure for adenine and hypoxanthine follows.

"18 ml. were evaporated to dryness in a lipped 200 × 25 mm. test tube for the adenine determination.[14] The purine hydrochlorides were taken up in 4 ml. of hot water; the solution was cooled, and 2 ml. of acidified sodium picrate solution were added.[15] The solution was filtered immediately through a paper pulp mat in a small bore filtration tube,[16] and the test tube and precipitate were washed first with 2 ml., then with 1 ml. of a very slightly acid solution which was one-half saturated with sodium picrate.[15] The filtrate and washings were received in a second test tube, and after washing down the outside of the filtration tube and the walls of the test tube with water, this solution was set aside for the hypoxanthine determination. The adenine picrate precipitate was transferred to the test tube in which it had been precipitated by means of a stream of hot water, and this solution was titrated[17] at the boiling point with 0.02 normal, carbonate-free sodium hydroxide. 1 ml. of the alkali is equivalent to 1.4 mg. of adenine nitrogen.[18]

"3 ml. of 1 normal nitric acid were added to the adenine filtrate, and this solution was heated in a boiling water bath. The hypoxanthine and chloride were precipitated by adding 2 ml. of 0.2 normal silver nitrate solution. The heating was continued for 5 to 10 minutes, and then the solution was allowed to cool slowly to room temperature. When cool, the

[13] We prefer to filter the hot solution and wash the tube and filter with hot N HCl, and then after cooling dilute to 25 cc. Less adsorption of purines by the Cu_2S occurs in the presence of hot N HCl than in the cold diluted acid.

[14] The evaporation is made by means of a current of hot air while the tube is immersed in hot water (Logan, 1930). When the volume has been decreased to about 5 cc., the flame under the water bath is removed, so that the temperature at the end of the evaporation is about 30°.

[15] Since adenine picrate is soluble in alkaline picrate, the sodium picrate is acidified (methyl orange indicator) with picric acid.

[16] See Fiske and Logan (1931).

[17] Titrate to the first color change of the indicator (2 drops of 0.05 per cent phenol red, neutralized).

[18] We titrate with the same 0.01 N NaOH as used for the micro-Kjeldahl determination. 1 cc. of this is equivalent to 0.7 mg. of adenine nitrogen.

solution was filtered through asbestos in a 2.5 cm. filtration tube,[19] the greater part of the precipitate having been retained in the test tube. The precipitate was washed by decantation with three 5 ml. portions of water. The hypoxanthine silver picrate was dissolved by 3 ml. of hot concentrated nitric acid, and this solution was poured through the filter, and was received in a third large pyrex test tube. The test tube in which the precipitation had been carried out and the filter were washed with three more 3 ml. portions of hot nitric acid. After the addition of 1 ml. of 10 normal sulfuric acid, the combined filtrate and washings was evaporated to a small volume, and the organic matter was ashed. 3 ml. of water, and 1 ml. of saturated ferric alum solution were added, and the solution was titrated with 0.01 normal ammonium thiocyanate solution. 1 ml. of the thiocyanate is equivalent to 0.56 mg of hypoxanthine nitrogen."

Results obtained by the use of the micro- and macroprocedures for nucleotide are close, and agree with those obtained with the older procedure (Kerr and Blish, 1932) when applied to extracts of fresh tissues (Table I). The results are more divergent when autolyzed tissues are analyzed (Table I). According to Peham (1937) high results are obtained with some tissues by the Kerr-Blish procedure on account of the large amounts of uranium phosphate and copper hydroxide which are precipitated. This does not appear to be true for fresh muscle, brain, or blood. The time required for a series of determinations is much less than with the older procedure. Four determinations (micro) in duplicate may be completed within 3 hours after the neutralized trichloroacetic acid extract of the tissue is available.

The recovery of nucleotide added to tissue extracts was determined by both the micro- and macroprocedures. Portions of analyzed solutions of the sodium salt of adenosine triphosphate[20] were added to neutralized trichloroacetic acid extracts of dog muscle, the original composition of which was determined simultaneously. Recovery varied from 91.5 to 100.6 per cent by the microprocedure, and 98.8 to 100.6 per cent by the macromethod (Table I).

The recovery of nucleoside by the revised microprocedure was

[19] A mat of asbestos pulp on a small sintered glass filter serves equally well.

[20] Prepared from a pure dibarium salt by treatment with sodium sulfate solution. The barium salt was prepared by a procedure to be published in the near future.

Table I

Comparison of Determinations of Nucleotide and Nucleoside Plus Free Purine by Revised Micro-, Macro-, and Kerr-Blish Procedures. Recovery of Added Nucleotide and Nucleoside (Mg. per 100 Gm. of Tissue)

All values for the microprocedures represent the average of duplicate determinations. In Experiments 27-12 and 27-17 additional pairs of determinations were made to determine how closely results are duplicated. All tissue specimens were taken from dogs under amytal anesthesia.

Experiment No.	Tissue	Nucleotide N			Nucleoside + free purine N		Purine added	Per cent recovery	
		Kerr-Blish	New micromethod	Macromethod	Kerr-Blish	New micromethod		New micromethod	Macromethod
27-12	Muscle		46.2	46.7			Na adenosine triphosphate	93.1	100.6
			46.4					100.6	
			45.7					99.4	
27-13	"		23.4	22.9			" "	91.5	98.8
27-17	"	35.3	36.7	34.0			" "	93.6	
			35.3					101.2	
17-61	Brain		18.8			3.2	Adenosine	97.3	
							Guanosine	90.2	
27-20	Muscle (1 hr. post mortem)	36.2	38.6	31.8	5.8	6.0			
27-22	Muscle (autolyzed 24 hrs. at 38°)	6.2	6.2	4.5	10.3	9.0			
27-18	Liver (fresh)	30.4	31.2	27.0	1.7	3.8			
	Same liver autolyzed 24 hrs. at 38°	Lost	7.0	5.9	53.6	56.0			
27-21	Liver	22.9	22.8	21.9	2.3	3.0			
	Same liver autolyzed 24 hrs. at 38°	12.2	9.6	7.9	59.6	54.5			
25-11A	Brain	19.2		20.5					
25-9	"	21.8		21.7					
25-14	"	14.8		14.4					
27-19	"	19.4	19.3	18.2	3.0	2.6			
27-25	Blood	2.7	2.6		0.2	0.3			

also determined. Powdered frozen brain was added to three flasks containing 10 per cent trichloroacetic acid, one of them containing adenosine and another guanosine. Analysis of the three extracts showed a recovery of 92.3 per cent in the case of adenosine and 90.2 per cent for guanosine.

We find that nucleoside is not quantitatively precipitated with copper hydroxide; hence the hydrolysis in normal acid cannot be omitted when the filtrate from the uranium nucleotide precipitate is analyzed.

DISCUSSION

In studies of nucleotide metabolism it is desirable to know the extent of deamination of adenine and also the number of phosphate groups attached to the nucleoside. The first of these two objectives has been accomplished in the extension of our method to include the separate determination of adenine and hypoxanthine by Hitchings' procedure. Unfortunately the uranium precipitate is not suitable for measuring the amount of hydrolyzable organic phosphorus precipitated with the nucleotide. In the search for other precipitants mercuric acetate was studied, since Fiske and Subbarow (1929, *b*) used this reagent in the preparation of adenosine triphosphate and stated the yield to be not far from quantitative. Moreover this precipitate can be used readily for the determination of the various phosphorus fractions after removal of the mercury by H_2S while cold. When applied to the neutralized trichloroacetic acid extracts of tissues, however, only 20 to 30 per cent of the adenosine triphosphate present is precipitated by 0.05 volume of 20 per cent mercuric acetate in the presence of 2 per cent acetic acid. When the acidity is reduced to 0.1 per cent, the precipitation is quantitative, but nucleosides and purines are also precipitated (adenosine quantitatively, hypoxanthine 87 to 90 per cent). Hence mercuric acetate cannot be used in place of uranium acetate to separate nucleotides from nucleosides and free purines.

Although the major portion of the nucleotide of fresh tissue is adenosine triphosphate, certain other compounds discovered since the publication of our procedure in 1932 are probably also precipitated. Diphosphopyridine nucleotide is stated by Myrbäck and Örtenblad (1935) to be precipitated by uranyl acetate, at

least from purified solutions. Presumably the closely related triphosphopyridine nucleotide (Warburg and Christian, 1936) is likewise precipitated. We do not know what proportion of the total adenine is split from these compounds during the 20 minute hydrolysis period. We have ascertained that nicotinic acid amide, if set free, is not precipitated by copper and bisulfite under the conditions used for precipitating purines. The quantity of adenine combined in the pyridine nucleotides may be estimated for a number of tissues from the data given by Karrer and Keller (1938). From their values for nicotinic acid amide in muscle we calculate the equivalent in adenine nitrogen to be 0.012 mg. of N per 100 gm., whereas mammalian muscle contains from 40 to 50 mg. of acid-soluble nucleotide nitrogen.[21] Hence only 0.03 per cent of the nucleotide purine in muscle is present in the pyridine nucleotide. The figure given by Ochoa (1937) for diphosphopyridine nucleotide in rabbit muscle is equivalent to 0.006 mg. of adenine N per 100 gm. Similar calculations from the data of Karrer and Keller (1938) indicate that liver contains 0.07 mg. of adenine in the form of pyridine nucleotide, roughly 0.1 per cent of the value found by Dell'Acqua (1935) for nucleotide plus nucleoside and free purine N in rabbit liver. These amounts are obviously too small to be measured by our procedure, since they are exceeded by the error of the method. Horse erythrocytes, however, are reported to contain 12 mg. of triphosphopyridine nucleotide per liter (Warburg, Christian, and Griese, 1935), a value which corresponds to 0.10 mg. of adenine N per 100 cc. of cells, the total nucleotide N of which amounts to 1.13 mg. (Kerr and Daoud, 1935). In this case the adenine combined in the pyridine nucleotide appears to represent about one-tenth of the total.

SUMMARY

The micromethod for determining purine nucleotides published by Kerr and Blish (1932) has been simplified and shortened. An adaptation of this procedure permitting the separate estimation of adenine and hypoxanthine by the procedure of Hitchings (1933) is also presented.

[21] Unpublished data.

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RACEMIZATION OF AMINO ACIDS AND DIPEPTIDES ON ACETYLATION WITH KETENE*

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A method of acetylation that has assumed prominence of late involves the use of ketene. Although it reacts with water to form acetic acid, ketene combines preferentially with the α-amino group of an amino acid dissolved in water, and it was shown by Bergmann and Stern (2) that amino acids may be conveniently acetylated with this reagent. Because of the simplicity and effectiveness of the reaction, ketene has also been used to acetylate such physiologically active proteins as pepsin (3) and insulin (4). In a recent investigation by Jackson and Cahill (5) it was observed that under certain conditions an optically active amino acid, when acetylated, yielded a completely racemized acetyl derivative. In the present study, observations on the use of ketene as an acetylating agent, with particular attention to its racemizing action, were extended to other representative amino acids as well as to dipeptides. The racemizing action of ketene was also compared with that of acetic anhydride.

$l(-)$-Leucine as well as the dicarboxylic amino acid, $l(+)$-glutamic acid, can be conveniently acetylated to yield either the optically active or racemized acetylated product, depending upon the experimental conditions. As was observed by Jackson and Cahill (5), if the solution of an amino acid is kept continuously alkaline to phenolphthalein during the introduction of ketene gas, no racemization occurs; however, if ketene is allowed to generate acetic acid in excess of the alkali present, rapid racemization results. An unsuccessful attempt was made to acetylate $l(-)$-

* A preliminary report was presented before the Thirty-third meeting of the American Society of Biological Chemists at Toronto, 1939 (1).

cystine under racemizing conditions. Free sulfur appeared in the course of the reaction and no uniform acetylated product could be obtained. Du Vigneaud and Meyer (6) have reported the splitting out of free sulfur on treating diacetyl-*l*(−)-cystine with acetic anhydride. The racemizing action of ketene on the basic amino acids, arginine and histidine, has been reported by Neuberger (7).

Information as to the rate of racemization was obtained by dissolving acetyl-*l*(−)-tryptophane in alkali and passing ketene through the solution. The change in optical activity with respect to time is shown on Fig. 1. The pH of the solution was also followed by means of a glass electrode and it was observed that as long as the pH remained on the alkaline side of neutrality no racemization occurred, whereas after the solution became acid, rapid racemization resulted. For purposes of comparison acetyl-

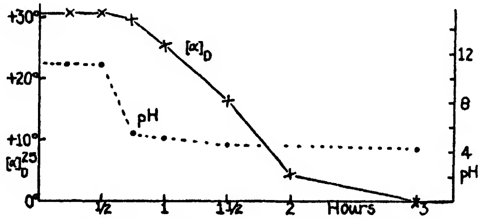


Fig. 1. Rate of racemization of acetyl-*l*(−)-tryptophane on treatment with ketene and the pH of the solution at various time intervals.

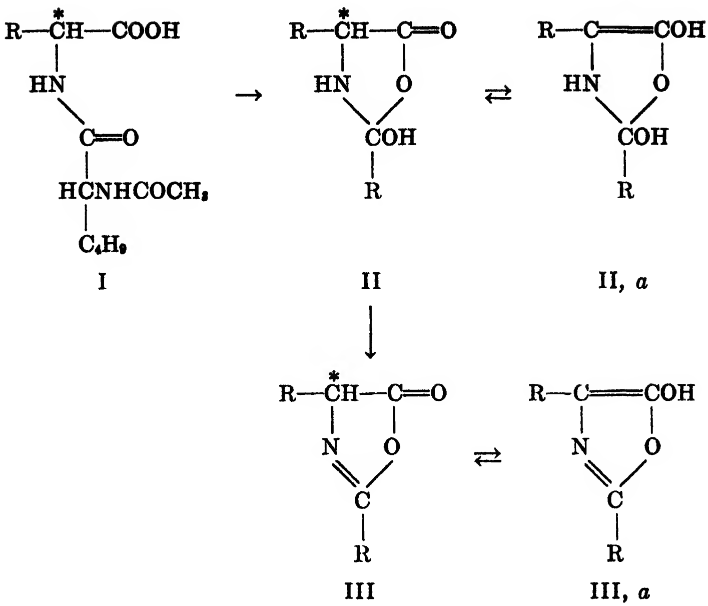
l(−)-tryptophane was also treated with acetic anhydride under conditions which were shown by du Vigneaud and Sealock (8) to cause racemization, and it was found that complete racemization was effected within 15 minutes.

The mechanism of racemization by acetic anhydride has been studied by Bergmann and Zervas (9), who considered several possible mechanisms and believed the reaction to be catalytic, and by du Vigneaud and Meyer (10) who emphasized the temporary formation of an azlactone ring in the racemization process. With regard to racemization with ketene, Jackson and Cahill (5) found that although acetyl-*l*(−)-proline could not be racemized by this reagent, racemization of acetyl-N-methyl-*l*(−)-tryptophane, which like proline contains an alkylamino group, could be effected. In this latter case the formation of an azlactone, with the amino nitrogen bearing a double bond, was not possible.

Extremely interesting results were obtained when ketene was utilized to acetylate dipeptides. Glycyl-*l*(−)-leucine and *l*(−)-leucylglycine were chosen for these experiments because in one dipeptide the optically active amino acid is in the terminal position, while in the other glycine is in this position. It was found that when either of these dipeptides was acetylated with ketene in alkaline solution, the optically active acetyl derivative of each dipeptide could be readily obtained. When, however, the dipeptides were acetylated under racemizing conditions, that is, when the solution was not kept alkaline during the course of the reaction, the acetyl derivative of glycyl-*l*(−)-leucine obtained was completely inactive, whereas that of *l*(−)-leucylglycine retained its full optical activity.

This selective racemization with ketene is in contrast to the racemizing action of acetic anhydride on a dipeptide, observed by Bergmann and Zervas (9). These investigators found that at least partial racemization of both amino acids in a dipeptide occurred on acetylation with acetic anhydride under the conditions used by them. In the study of Bergmann and Zervas, however, acetylation was effected by acetic anhydride in glacial acetic acid; *i.e.*, with the exclusion of water. It was, therefore, thought worth while to study the acetylation of dipeptides with acetic anhydride in aqueous solution inasmuch as acetylation by ketene was effected under these conditions. In these experiments glycyl-*l*(−)-leucine and *d*(+)-leucylglycine were acetylated by acetic anhydride in aqueous solution in the manner used by du Vigneaud and his collaborators (6, 8, 10) to prepare the optically active as well as the racemic derivatives of amino acids. Results were obtained which are similar to those procured with ketene. In the case of glycyl-*l*(−)-leucine either the active or racemic acetyl derivative can be prepared, depending upon the conditions employed. On the other hand, only the active acetyl derivative can be prepared from *d*(+)-leucylglycine.

The possible mechanisms formulated by Bergmann and Zervas and by du Vigneaud and his collaborators to explain the racemization of amino acids on acetylation with acetic anhydride may be applied to the racemization of dipeptides on acetylation with either ketene or acetic anhydride in the manner shown in the accompanying reactions.



The cyclic form (II) of the acylated dipeptide may be in equilibrium with the ring compound (II, *a*) or the azlactone (III) may be formed which is in equilibrium with its enol form (III, *a*). In either case the asymmetry of the active carbon would be lost. The formulas would explain why the terminal amino acid in glycyl-*l*(−)-leucine may be racemized when treated with ketene or acetic anhydride under the proper conditions, while the *l*(−)-leucine in *l*(−)-leucylglycine, not being in the terminal position and, consequently, not having a free carboxyl group, undergoes no racemization. The selective racemizing action of ketene, as well as of the more common anhydride of acetic acid, may prove to be a convenient tool for identifying terminal amino acids in peptides.

EXPERIMENTAL

Acetyl-l(−)-Leucine—300 mg. of *l*(−)-leucine[1] ($[\alpha]_D^{27}$ = +16.5° in 20 per cent HCl) were dissolved in 2 cc. of 2.5 N NaOH in a

[1] For this and other amino acids thanks are expressed to Dr. Melville Sahyun of Frederick Stearns and Company.

15 cc. centrifuge tube, and a slow stream of ketene gas was passed through the solution for 30 minutes, at the end of which time 1 cc. of 2.5 N NaOH was added to keep the solution alkaline. The gas was passed through the solution for ½ hour longer. At this point the solution was alkaline to phenolphthalein. Dilute sulfuric acid exactly equivalent to the sodium present was added and the solution was evaporated to dryness in a vacuum desiccator. The residue was extracted with acetone. On evaporation of the acetone solution, 260 mg. of crystals were obtained, which on recrystallization from water yielded small bars melting at 186°.[2] For $C_8H_{15}O_3N$ the calculated N value is 8.09; found, 8.00, 8.04 per cent. $[\alpha]_D^{29} = -21.0°$ (3 per cent solution in ethanol). Cherbuliez *et al.* (11) report a melting point of 181° and $[\alpha]_D^{29} = -16.99°$ (in alcohol) for an active acetylleucine obtained from a protein hydrolysate in which the amino acids had been acetylated, esterified, and subsequently separated by a procedure involving fractional distillation. The active acetylleucine obtained by these investigators was apparently partially racemized.

Acetyl-dl-Leucine—300 mg. of *l*(−)-leucine were dissolved in 2 cc. of 2.5 N NaOH and acetylated with ketene as above, except that at the end of 1 hour the reaction was not terminated but ketene was allowed to pass through the solution at double the initial rate for 1 hour longer. Shortly after the first hour the solution became acid to litmus and after 2 hours the pH was 4.5. After recrystallization, 250 mg. of large flat needles melting at 160–161° were obtained. Fischer (12) reported 161° for acetyl-*dl*-leucine. The substance was completely devoid of optical activity.

Acetyl-l(+)-Glutamic Acid—1 gm. of *l*(+)-glutamic acid ($[\alpha]_D^{24} = +32.1°$ in N HCl) was dissolved in 6 cc. of 2.5 N NaOH and ketene was passed through the solution for 1 hour, at the end of which time the solution was still alkaline to phenolphthalein. Dilute H_2SO_4 exactly equivalent to the alkali originally present was added and the solution taken to dryness in a vacuum desiccator over KOH and $CaCl_2$. The residue was extracted with ethanol, the alcoholic solution taken to dryness, and the crystals obtained recrystallized from a minimum amount of water. 780 mg. of large prisms, melting at 198–199°, were obtained in two

[2] All melting points are corrected.

crops. $[\alpha]_D^{24.5} = +3.9°$ (102.6 mg. in 5 cc. of N NaOH). Bergmann and Zervas (9) reported a melting point of 199° and $[\alpha]_D^{22} = +3.83°$ in N NaOH for acetyl-*l*(+)-glutamic acid.

Acetyl-dl-Glutamic Acid—1 gm. of *l*(+)-glutamic acid was dissolved in 2.5 N NaOH and the amino acid was treated with ketene as in the preparation of the active derivative, except that the ketene was passed into the solution at such a rate that it became acid to litmus in 45 minutes. The gas was then passed through the solution at twice this rate for 1½ hours longer. 900 mg. of small irregular prisms melting at 182–183° were obtained. Bergmann and Zervas (9) reported 180° (uncorrected) for acetyl-*dl*-glutamic acid. The material was completely optically inactive.

Determination of Rate of Racemization of Acetyl-l(−)-Tryptophane—246 mg. of acetyl-*l*(−)-tryptophane were dissolved in 6 cc. of N NaOH and a stream of ketene gas was passed through the solution for 15 minutes. After determination of the pH by means of a glass electrode, the solution was made acid to Congo red and then let stand in the refrigerator for a few hours. The optical activity and the melting point of the acetyltryptophane obtained were determined. Several portions of acetyltryptophane were treated in the same manner, except that the reaction was interrupted after 30, 45, 60, 90, 120, and 180 minutes, respectively. The pH of the solution at these time intervals and the rate of racemization of acetyl-*l*(−)-tryptophane treated in this manner are shown in Fig. 1. The optical activity of the acetyltryptophane obtained after a 15 minute treatment with ketene was $[\alpha]_D^{25} = +30.8°$ (46.3 mg. in 5 cc. of 0.1 N NaOH) and the melting point was 189–190°; after a 3 hour treatment the product obtained was devoid of optical activity and melted at 206°. Du Vigneaud and Sealock (8) report $[\alpha]_D^{31} = +29°$ (1 per cent solution of sodium salt), m.p. 189–190°, for acetyl-*l*(−)-tryptophane, and m.p. 205–206° for acetyl-*dl*-tryptophane. For comparison acetyl-*l*(−)-tryptophane was treated with acetic anhydride according to the procedure of du Vigneaud and Sealock (8). Complete racemization was found to be effected within 15 minutes.

Acetyl-l(−)-Leucylglycine—300 mg. of *l*(−)-leucylglycine (Schuchardt) were dissolved in 2 cc. of 2.5 N NaOH and ketene was passed through the solution for ½ hour. 1 cc. of 2.5 N alkali was added to keep the solution alkaline and the treatment with

ketene was continued for ½ hour longer. The pH at this time was 11.2. Dilute H_2SO_4 exactly equivalent to the base present was added and the solution was taken to dryness in a vacuum desiccator over KOH and $CaCl_2$. The residue was extracted with acetone and the acetone solution concentrated until incipient crystallization occurred. After having stood in the refrigerator for several hours, the solution was filtered and 205 mg. of colorless broken needles melting at 193–194° were obtained, which gave $[\alpha]_D^{26} = -43.2°$ (52.1 mg. in 5 cc. of H_2O). For $C_{10}H_{18}O_4N_2$ the calculated N value is 12.17; found, 12.08, 12.40 per cent.

Similarly, acetyl-*d*(+)-leucylglycine could be obtained by acetylation of *d*(+)-leucylglycine with acetic anhydride according to the acetylation procedure used by du Vigneaud and Sealock (8) to prepare acetyl-*l*(−)-tryptophane. The material was isolated as above. The product melted at 193–194° and exhibited $[\alpha]_D^{20} = +43.7°$ (1 per cent aqueous solution).

Acetylglycyl-l(−)-Leucine—300 mg. of glycyl-*l*(−)-leucine (Schuchardt) were acetylated as above. 260 mg. of colorless crystals melting at 129° were obtained. $[\alpha]_D^{26} = -25.3°$ (52.3 mg. in 5 cc. of H_2O). Bergmann and Zervas (9) reported $[\alpha]_D^{18} = -25.62°$, m.p. 129–130°, for acetylglycyl-*l*(−)-leucine. Acetylglycyl-*l*(−)-leucine could also be obtained by acetylation with acetic anhydride according to the acetylation procedure of du Vigneaud and Sealock referred to above. The melting point was 129° and a mixed melting point with the product prepared by acetylation with ketene showed no depression. $[\alpha]_D^{20} = -25.3°$ (1 per cent aqueous solution).

Acetylglycyl-dl-Leucine—300 mg. of glycyl-*l*(−)-leucine were dissolved in alkali and acetylated with ketene as above. In this case, however, the ketene gas was passed in at such a rate that the solution became acid to litmus in 1 hour, and the treatment with ketene was allowed to continue for an additional hour. The pH at this time was 4.4. 180 mg. of colorless platelets melting at 177° and possessing no optical activity were obtained. The melting point of acetylglycyl-*dl*-leucine was reported to be 177° by Bergmann and Zervas (9). Acetylglycyl-*dl*-leucine could also be prepared by acetylation of glycyl-*l*(−)-leucine with acetic anhydride according to the method used by du Vigneaud and Sealock (8) to prepare acetyl-*dl*-tryptophane from *l*(−)-trypto-

phane. The product obtained was devoid of optical activity. The melting point was 177° and a mixed melting point with the product prepared by acetylation with ketene showed no depression.

Attempted Racemization of Acetyl-l(−)-Leucylglycine—300 mg. of *l*(−)-leucylglycine were treated with ketene in the same manner as glycyl-*l*(−)-leucine in the preceding experiment. The pH of the solution after treatment with ketene was 4.4. 250 mg. of colorless broken needles melting at 193–194° were obtained. A mixed melting point with the acetyl-*l*(−)-leucylglycine described earlier in this paper showed no depression. $[\alpha]_D^{26} = -43.5°$ (51.2 mg. dissolved in 5 cc. of H_2O). Another similar run yielded a product with $[\alpha]_D^{25} = -43.3°$. Thus under conditions of acetylation sufficient to racemize completely acetylglycyl-*l*(−)-leucine, *l*(−)-leucylglycine yields acetyl-*l*(−)-leucylglycine of maximal optical activity.

Similarly, treatment of *d*(+)-leucylglycine with acetic anhydride in the manner which was found to racemize completely glycyl-*l*(−)-leucine resulted in the production of acetyl-*d*(+)-leucylglycine. The product obtained showed $[\alpha]_D^{30} = +43.7°$ and melted at 193–194°.

SUMMARY

l(−)-Leucine and *l*(+)-glutamic acid can be acetylated with ketene to give either the optically active or racemic N-acetyl derivative, depending upon the conditions employed.

A study of the rate of racemization of acetyl-*l*(−)-tryptophane by ketene shows that the racemization takes place only when the pH of the solution is on the acid side of neutrality.

Both glycyl-*l*(−)-leucine and *l*(−)-leucylglycine can be readily acetylated with ketene to give the corresponding optically active acetyl derivative. When acetylated under racemizing conditions, however, glycyl-*l*(−)-leucine yields the completely racemized derivative, whereas under the same conditions *l*(−)-leucylglycine yields acetyl-*l*(−)-leucylglycine of maximal optical activity. Similar results are obtained with acetic anhydride under the proper conditions.

It is pointed out that the selective racemization observed might serve as a convenient tool for identifying the terminal amino acid in peptides.

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ON THE ISOLATION OF A GLUCOSE-CONTAINING CEREBROSIDE FROM SPLEEN IN A CASE OF GAUCHER'S DISEASE*

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In his recent book Page (1) has described the past and recent work on cerebrosides and the present status of knowledge concerning these compounds. He states, "Cerebrosides are of glucosidic nature and, on hydrolysis, yield a reducing sugar (galactose), a base (sphingosine) and a fatty acid." The only variations in the cerebrosides have been considered to be due to the fatty acid: phrenosin, kerasin, and nervon containing cerebronic, lignoceric, and nervonic acids, respectively. These compounds are found normally in the brain, but in Gaucher's disease the spleen becomes greatly enlarged due to a deposit of cerebroside.

In the analyses of such spleens emphasis has largely been on the identification of the cerebroside as kerasin or phrenosin by combustion analysis, by specific rotation in various solvents, by the selenite test of Rosenheim (2), or by the melting point and molecular weight of the fatty acid obtained after hydrolysis. Less attention has been given to the nature of the carbohydrate, probably because extensive work on cerebroside from brain has amply demonstrated the presence of galactose in this compound. Lieb (3) identified the carbohydrate from spleen in a case of Gaucher's disease as galactose by the osazone. McConnell and coworkers (4) state that, "after acid hydrolysis, sphingosine sulfate, galactose and a fatty acid. . .were obtained," without, however, giving the method used to identify the carbohydrate. Capper and coworkers

* The results reported here were given at the meeting of the Society for Experimental Biology and Medicine at Palo Alto, June 30, 1939.

(5) stated that kerasin and a small amount of phrenosin were obtained from a spleen which they analyzed.

In the spleen which we obtained and on which we wish to report, glucose, rather than galactose, was found as the component carbohydrate, although the cerebroside in other respects closely resembled kerasin. Because of this unusual finding it appears of interest to report briefly the analytical procedures followed, the methods used to identify the various compounds obtained, and the results on which our conclusions are based.

EXPERIMENTAL

The spleen, which weighed about 1500 gm., was removed at operation on October 28, 1938. It was held in a formalin fixative until December 16, when a sample was removed for analysis. A second sample was obtained 2 months later, but in this interval of time considerable decomposition of the tissue had taken place. As a check on the methods of analysis used, and in order to determine whether the fixative could, in some unknown manner, have affected the carbohydrate, comparative studies were made on a normal human brain, part of which was analyzed fresh and the remainder after 6 weeks in a similar formalin preservative.

Extraction and Purification of Cerebrosides—The first sample of spleen was dried to constant weight in a vacuum oven at 45°. Two 24 hour extractions with ether were then carried out to remove lipids. The tissue was dried and pulverized and extracted repeatedly with hot 95 per cent alcohol until no further cerebroside was obtained. After filtration of the cerebroside from the cooled alcoholic extract, it was dissolved in pyridine and precipitated with acetone and then recrystallized from alcohol-chloroform. The second sample of spleen and both brain samples were dehydrated with acetone (which process also removed sterols and lecithin). After the tissues were dried *in vacuo*, they were extracted with ether and then hot alcohol and the cerebrosides were purified as above. These ether extracts were concentrated, and when cooled a considerable precipitate was obtained. Since ether has been found to extract some cerebroside from brain (1), the precipitates were also carried through the pyridine-acetone purification and recrystallized from alcohol-chloroform.

Identification of Compounds—The cerebrosides were identified

by solubilities, melting points, and nitrogen content. Determinations of total phosphorus were made on the purified materials as a means of ascertaining how much the cerebroside was contaminated with sphingomyelin. Because of similar physical properties it is practically impossible to separate this phosphatide completely from the cerebrosides. After hydrolysis, the acids obtained were identified by melting points, molecular weights, and solubilities, sphingosine sulfate by its nitrogen content and melting point, and the carbohydrates by fermentation tests and the melting points of osazones. In each melting point determination this temperature was raised at the rate of 1° every 2 or 3 seconds, as recommended by Morrow and Sandstrom (6). The mucic acid test for galactose was carried out on two samples of the carbohydrate from spleen, according to the method given by the above authors. In order to determine whether there might be a β-glucoside linkage in the cerebroside, samples from both brain and spleen were treated with emulsin. Since glucose, fructose, and mannose are all fermentable and form the same osazone, it was necessary to determine which of these sugars was present, and to this end the hydrolysates were treated with microorganisms, according to the method of Harding and Nicholson (7). For this work it was necessary to use the cerebrosides obtained from the ether extracts, since the supply of the original material was exhausted. However, as one can see from Table I, the compounds are identical.

Analytical Methods

Hydrolysis—Attempts were made to hydrolyze the cerebrosides with hydrochloric acid. However, neither the use of 0.6 N aqueous acid for $2\frac{1}{2}$ hours nor of 5 per cent acid in absolute alcohol for 7 hours yielded any appreciable amount of sugar. We therefore followed the method of Rosenheim (8) or of Tropp and Wiedersheim (9) and hydrolyzed the cerebrosides with 10 per cent sulfuric acid in methyl alcohol for 6 hours or with 2.5 per cent acid for 12 hours. The process was carried out on a water bath under a reflux condenser. Large losses of carbohydrate resulted when this method was employed, as shown by the low yield after hydrolysis of the cerebrosides. When glucose and galactose were subjected to such treatment, only about 70 per cent could be

recovered. Yields more nearly approaching theoretical were obtained by the micromethod of Kirk (10) in which the material

TABLE I

Analyses of Spleen from a Patient with Gaucher's Disease Compared with Analyses of Normal Brain

	Spleen		Brain	
	7 wks. in formalin	21 wks. in formalin	Fresh	6 wks. in formalin
Moisture, % *fresh weight*	78.1		68.0*	
Acetone-soluble material, % *fresh weight*		79.6	89.0	76.5
Ether-soluble material, % *fresh weight*	1.7	1.0		8.8
Cerebrosides				
Alcoholic extract (after ether extraction), % *dry weight*	14.9	8.5	8.5	5.8
Ether extract, *gm.*		0.331		2.15
M.p. (178°)†				
Alcoholic extract, °*C.*	175, 176	174	174	176, 178
Ether extract, °*C.*		175		178, 179
Nitrogen (1.73)†				
Alcoholic extract, %	1.71, 1.74	1.60, 1.67	1.90, 2.00	1.91
Ether extract, %		1.70		1.91, 2.00
Total P, %		0.25	1.24	0.7
Hydrolysates				
Sphingosine sulfate				
Nitrogen (3.26),† %		3.00‡		3.00‡
M.p. (242–245°),† °*C.*		248–250‡		248–250‡
Fatty acids, ester % of cerebroside (45.5)†	32.4 (5)	36.6 (3)	54.4 (2)	49.7 (2)
M.p. (lignoceric acid 81°, cerebronic acid 101°)				
Recrystallized from methyl alcohol, °*C.*	78 (2), 77 (2)	76 (1), 77 (2)	96 (4)	94 (4)
Recrystallized again from acetone, °*C.*		76 (2)		90 (2)
Mol. wt. (lignoceric acid 386, cerebronic acid 408)	384 (2)	370 (2)	422 (2)	441 (2)

TABLE I—*Concluded*

	Spleen		Brain	
	7 wks. in formalin	21 wks. in formalin	Fresh	6 wks. in formalin
Hydrolysates—concluded				
Carbohydrate				
% of cerebroside, maximum recovery (22.2)†				
Alcoholic extract Rosenheim method...........	15.0	2.2	9.2	5.8
Kirk method......		17.9		15.0
Ether extract, Kirk method		17.8		24.0
Loss on fermentation				
Alcoholic extract,%.	92 (2)	100 (3)	8 (3)	3 (3)
Ether extract, %..		100 (3)		7.4 (3)
Mucic acid test... ...	Negative (2)			
Osazone, m.p., (glucosazone 210–212°, galactosazone 196°)†..	207–208	210	198	194
Mixed with glucosazone, °C..	208–209	210–212		
Mixed with galactosazone, °C	204–205			196
Hydrolysis by emulsin				
Sugar in filtrate, %.		2.5		0.8
" " residue, %.		2.8		6.9

The numbers in parentheses in the last four columns indicate the total number of samples analyzed.

* Determined on an aliquot of the fresh material.

† Theoretical value.

‡ Mixed sample used.

is hydrolyzed for only 10 minutes with 3 N hydrochloric acid. This latter method allows only for determination of carbohydrate, but it was also used satisfactorily as a means of confirming conclusions concerning the nature of the monosaccharide.

Fatty Acids (*and Esters*)—After hydrolysis the solutions were chilled, and the fatty acid esters removed by filtration, dried in a vacuum desiccator, and weighed. They were then saponified and

reacidified, and the acids extracted with ether. The ether was washed, dried, and then evaporated, and the acids were recrystallized twice from dry methyl alcohol and in some cases twice more from redistilled acetone.

The molecular weights of the acids were determined by saponification in dilute alcoholic potassium hydroxide, the excess alkali being titrated against dilute sulfuric acid. The acids could be recovered by acidifying (after the end-point had been reached) and extracting with ether. After recrystallization, the acid could be used for a second determination.

Sphingosine—After removal of the lipid material, water was added to the hydrolysates and the solutions boiled 1 hour to hydrolyze any esters present, after which the alcohol was distilled off. The sphingosine was removed with difficulty. If the material was kept ice-cold, the greater part could be filtered off slowly. No suction could be applied, since the sphingosine was drawn through the filter paper. Attempts were made to extract the material by means of ether, petroleum ether, or chloroform, but no method found would remove the material completely. The sphingosine sulfate obtained was dissolved in alkali, taken up in ether, and acidified, and the resulting material recrystallized from methyl alcohol. In no case was a quantitative yield obtained, and the material was very difficult to purify. However, a sample from brain cerebroside was obtained which melted at 248-250° (theoretical 242–245°), and a mixed sample from spleen and brain contained practically the theoretical nitrogen content.

Carbohydrate—After removal of the sphingosine the filtrate was neutralized with either sodium hydroxide or with barium hydroxide followed by carbon dioxide gas to remove the last traces of barium. The carbohydrate was determined by the Shaffer-Hartmann technique. To determine loss on fermentation, yeast (which had been washed and centrifuged five times) was added to an aliquot of the solution; the material was held at 37° for 1 hour and then centrifuged, and determination was made of the non-fermentable sugar. When the Kirk method was used for hydrolysis, the colorimetric method of Jeghers and Myers (11) or the copper method of Harding and Downs (12) was employed. Both of these methods are more accurate for lower concentrations of sugar than the Shaffer-Hartmann.

The osazones were prepared by the usual procedures and repeatedly recrystallized from 60 per cent ethyl alcohol.

The emulsin was prepared from fresh almonds by the method of Tauber (13). Its activity was tested against salicin. For hydrolysis the dry cerebroside was added to a 1 per cent emulsin solution, acetate buffer at pH 4.4 was added, and the flasks held at 37° for 48 hours. It is possible that under somewhat different experimental conditions a greater degree of cleavage might have occurred, but since positive results were obtained it was not considered essential to study the matter further.

Final Identification of Monosaccharide—When samples of glucose, fructose, mannose, and galactose were carried through the microhydrolysis process of Kirk, only about 50 per cent of the

TABLE II

Results of Treatment with Proteus vulgaris

Materials	Before treatment	After treatment	Per cent loss
	mg.	*mg.*	
Glucose	1.44	0.72, 0.68	50
Fructose	1.44	1.35	7
Mannose	1.44	1.44	
Galactose	1.44	1.44	
Hydrolysate, spleen	0.80		100
" brain	0.95	0.80	15

Another experiment yielded similar results.

fructose could be recovered, whereas the other three sugars showed no loss. Deuel and Chambers (14) have presented evidence that fructose is completely destroyed by boiling ½ hour in 10 per cent hydrochloric acid, while this treatment has no effect on glucose. Since theoretical yields of sugar were obtained from the cerebrosides by the microprocess and up to about half by the 6 or 12 hour hydrolysis with sulfuric acid, it appeared certain that the carbohydrate present in the cerebroside from spleen could not be fructose. This conclusion was borne out by results of the experiments with *Proteus vulgaris*.

A pure strain of this organism was obtained and cultured according to the methods given by Harding and Nicholson (7). The results from the use of *Proteus vulgaris* are shown in Table II.

DISCUSSION

The cerebroside separated from the spleen of a patient with Gaucher's disease belongs to a new class of glycolipids which probably differ from previously described cerebrosides only because they contain glucose in place of galactose. In such properties as solubilities, nitrogen content, and melting point of the cerebroside itself as well as the nature of the acids obtained after hydrolysis the behavior was identical with that of kerasin. On the other hand the cerebroside prepared from fresh or preserved brain had all the classical properties of the galactolipids.

The proof that glucose is the component sugar is based on a number of tests. In the first place the carbohydrate obtained after the hydrolysis of each of ten individual samples of the spleen cerebroside (by either the macro- or microprocedure) was invariably completely fermentable by yeast, while the sugar from the cerebroside prepared from the fresh or preserved brain was in no case acted on by yeast. Secondly, glucosazone was prepared from the spleen hydrolysates; this was identified by its crystalline form, melting point, and mixed melting point with known glucosazone. The sugar from both samples of brain cerebrosides was identified as galactosazone. Moreover, the sugar from the spleen cerebroside failed to give a positive mucic acid test. Lastly, it was demonstrated that the sugar from the hydrolysate of the spleen was completely destroyed by a culture of *Proteus vulgaris*; in experiments performed simultaneously it was found that a known glucose sample was largely destroyed, while mannose, fructose, and galactose as well as the carbohydrate from the hydrolysates of the brain cerebrosides were not attacked by these microorganisms.

The presence of glucose in the spleen cerebroside cannot be traced to an impurity in the cerebroside preparations. Not only did the cerebrosides exhibit the properties and analyses attributed to this class of compounds but the galactolipids prepared by similar procedures from brain contained the galactose molecule while no trace of galactose was found in the spleen cerebroside. Moreover, the spleen cerebroside contained a maximum of 6 per cent of sphingomyelin (as determined by phosphorus content), which is the most likely contaminant because of its similar solubility. The latter lipid, however, does not contain any carbo-

hydrate component. Somewhat larger amounts of sphingomyelin were probably present in the brain cerebrosides, the values being 16 and 32 per cent respectively for the cerebrosides prepared from preserved and fresh samples. The lower results in the preserved samples are in line with the findings of one of us (15) that phospholipids are rapidly lost when the tissues are held in formalin.

That the cerebrosides investigated by us are essentially pure preparations is also evident from the recovery of approximately theoretical amounts of fatty acid and carbohydrate from the hydrolysates. This fatty acid in the spleen cerebroside had the melting point and molecular weight of lignoceric acid which is normally found in kerasin. Although the prolonged hydrolysis of the cerebroside with sulfuric acid was necessary to obtain sufficient fatty acids and sphingosine for their identification, it could not be employed for a quantitative recovery of the carbohydrate component. However, amounts of carbohydrate approaching the theoretical were recoverable by the micromethod of Kirk in which a 10 minute hydrolysis with hydrochloric acid is used. In spite of the fact that it was impossible to separate sphingosine quantitatively, this compound was qualitatively identified by solubilities, melting point of its sulfate derivative, and by the nitrogen content. Sphingosine sulfate separated as a black tar-like material which was very hydroscopic. After repeated crystallization from methyl alcohol, a small yield of crystalline material was obtained.

The possibility also exists that the preservative used might have altered an original galactose molecule in the spleen cerebroside in some way to change it to glucose. That such a criticism is not valid is evident from the fact that the galactose in the brain cerebroside was not altered by treatment with a similar preservative over the same length of time.

A difference in the cerebrosides is also apparent from their behavior with emulsin. The spleen cerebroside was hydrolyzed to an extent of over 50 per cent by treatment with this β-glucosidase while the brain cerebroside was not attacked.

In all probability the glucose-containing cerebroside is the result of an anomaly in carbohydrate metabolism rather than the characteristic of Gaucher's disease. There seems to be ample evidence of the identification of galactose as the carbohydrate component in at least one case of Gaucher's disease (3). Possibly instances

in which glucose replaces galactose in the brain cerebroside may eventually be noted.

It is suggested that the term "glycolipid" be reserved for those cerebrosides which have glucose as the component carbohydrate while "galactolipid" be used to describe the cerebrosides containing the galactose molecule.

SUMMARY

The cerebroside separated from the spleen of a patient suffering from Gaucher's disease resembled kerasin except that it contained glucose rather than galactose.

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A RAPID MICRO-KJELDAHL METHOD

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For the past 4 years we have been using a micro-Kjeldahl method which possesses virtues of simplicity, accuracy, and rapidity that have led to its adoption by a number of other laboratories. The frequency of inquiries about the method would seem to justify a description of the apparatus and procedure.

The most important features of the method are the use of a vacuum still, slightly modified from Krogh's (1934) ammonia still, titration with the Rehberg (1926) microburette, and measurement of small volumes with the Krogh-Keys syringe pipette (1931). The method is applicable to quantities of 20 mg. down to 0.01 mg. of total nitrogen in the sample. With suitable precautions the method can be applied to samples containing as little as 0.0001 mg. of nitrogen if the Teorell (1932) titration is used. With quantities of nitrogen equivalent to that ordinarily contained in 0.1 to 0.2 cc. of blood serum the method is not appreciably less accurate than the standard macro-Kjeldahl procedure.

Reagents (for 0.02 to 0.2 Cc. of Serum)—

For the digestion fluid 3 parts of concentrated N-free H_2SO_4 and 1 part of concentrated (85 per cent) H_3PO_4 were used.

0.2 N H_2SO_4.

1.0 N KOH.

30 per cent H_2O_2 (superoxol, or perhydrol).

Ammonia-free distilled water.

Alcoholic solution of methyl red (0.01 per cent).

Measurement of Sample—Samples of the order of 0.2 cc. are quickly measured out with the Krogh-Keys syringe pipette (1931)[1]

[1] The syringe pipettes may be obtained from the Workshop of Professor August Krogh, Juliane Maries Vej 32, Copenhagen, Denmark.

with an error no greater than ±0.1 per cent in the following manner. The syringe pipette is flushed with distilled water and emptied, so that the dead space of the tip contains only water and no air bubbles are present. The sample is then drawn into the syringe pipette and delivered into the digestion flask. The syringe pipette is then flushed out three or more times with distilled water, the washings being delivered into the digestion flask each time. The entire procedure takes between 1 and 2 minutes and the syringe pipette is then ready for the next sample. The accuracy of measurement is not appreciably affected by viscosity and red cells may be measured as readily as serum.

Digestion—Digestion is carried out in ordinary 100 cc. Kjeldahl flasks which have been fitted with standard taper, 24 to 40 mm., female, ground joints. If necessary, add distilled water to bring the volume to at least 5 cc. Add two glass beads and 1 cc. of the digestion mixture. Digest over a microburner for 10 minutes, remove the flame, and add 1 drop of 30 per cent H_2O_2. Continue digestion over the flame for 10 minutes more, cool, and add 20 cc. of NH_3-free distilled water. Other digestion mixtures may be used if desired, provided they are nitrogen-free. The present digestion mixture etches the flasks, but when they get bad the bottoms may be replaced at small cost. We have not found any other digestion mixture so rapid and trouble-free.

Digestion may be carried out in an ordinary hood without special precautions against the negligible amount of H_2SO_4 vapor. During digestion it may be necessary to provide agitation to prevent bumping. We use a simple rack carrying eight flasks (25 inches long by 6 inches wide), one leg of which is slightly shorter than the others. The rack may be rocked gently by hand or, better, by a small motor.

Distillation Apparatus—The distillation apparatus is shown in Fig. 1.[2] This apparatus is designed to carry out distillation in a partial vacuum; the continual admission of a small supply of air through the fine capillary resistance washes through to the receiver any ammonia which would otherwise tend to be trapped in the system and greatly shortens the time needed to transfer all the ammonia to the receiver. Thermometer tubing makes a suitable

[2] The apparatus may be obtained from Mr. E. F. Greinke, 37 Physics Building, University of Minnesota, Minneapolis.

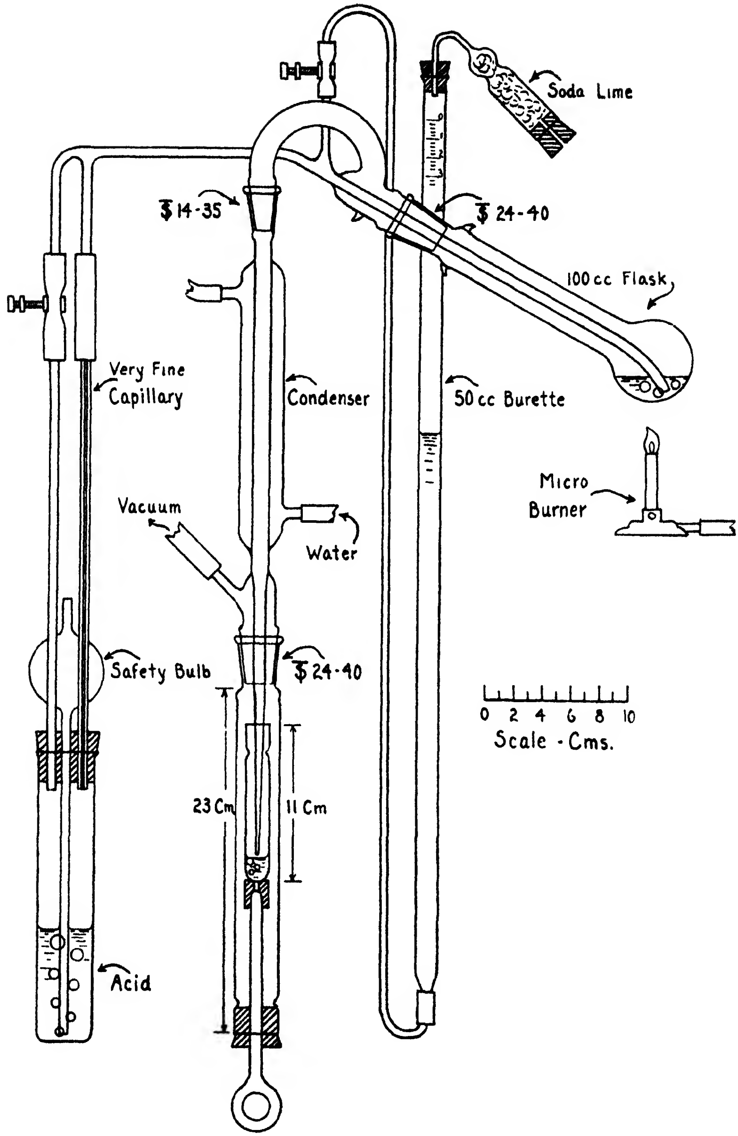

Fig. 1. Still for distillation of ammonia in a partial vacuum, showing complete apparatus in use during distillation.

capillary resistance. The air admitted is first bubbled through 10 per cent H_2SO_4 in the wash bottle. A screw-clamp on the second wide bore tube is provided to admit air to bring the system quickly back to atmospheric pressure when the distillation is completed.

The ground glass joints are lubricated with alkaline glycerol which is prepared by cooking six or seven KOH pellets (about 0.5 gm.) in 50 cc. of glycerol until the solution, when cooled, is a thick syrup. The hole in the rubber stopper which carries the glass rod supporting the distillate receiver is likewise lubricated with alkaline glycerol.

Provision is made for the rapid addition of a measured quantity of alkali to the digestion flask after the system is closed and at a partial vacuum by means of the screw-clamp on the line from the burette.

Distillation—The arrangement during distillation is shown in Fig. 1. At the start the screw-clamp on the alkali burette is closed, the screw-clamp on the air admission tube is open, and cold water is circulating in the condenser. The flask with the acid digest is put in place with a drop of alkaline glycerol on the ground joint. The air admission tube must reach nearly to the bottom of the flask. The standard acid (1 cc. of 0.2 N for most purposes) is placed in the receiving tube; this is inserted in its housing and adjusted by sliding the glass rod support until the acid level is about a cm. below the tip of the distillation line. Then the screw-clamp on the wide bore air admission tube is closed and the vacuum started. After a moment or so the receiver is adjusted so that the incoming air blows into the acid but does not cause it to splash. The proper amount of strong KOH (usually 5 cc.) is then admitted to the flask by opening the screw-clamp and distillation is begun.

As distillation proceeds, the receiver is lowered from time to time to keep the distillate at a level where there is no splashing. The vacuum should be sufficiently high so that rapid distillation takes place with the flask never more than comfortably warm to the touch. When about 8 cc. have been distilled, the burner is shut off and the neck of the flask and the connecting head warmed for a few moments by flaming them with a burner held in the hand. The receiver is then lowered well below the tip of the

condenser tube and the vacuum shut off. The system is quickly brought back to atmospheric pressure by carefully opening the screw-clamp on the wide bore air line. The receiver may then be removed. It should be stoppered until ready to titrate. The entire procedure of distillation takes from 6 to 10 minutes.

Titration—Titration is performed with the Rehberg microburette, 0.2 cc. capacity,[3] with 1 N KOH for all except the most extremely small amounts of nitrogen. The distillate receiving tube is hung in the forked support arm of the burette by means of the groove in the tube which also serves to prevent loss of the standard acid in the case of splashing either in distillation or titration. The air used for stirring in the titration is preferably washed through dilute KOH and dilute H_2SO_4 solutions. A standard amount of methyl red (*e.g.*, 2 small drops of the alcoholic solution) is used as indicator. We prefer to add the indicator to the acid in the receiving tube before distillation.

Calculation—The results are calculated in the ordinary manner.

$$\text{Mg. N in sample} = 14 \times (\text{cc. of acid} \times \text{normality of acid minus cc. of titration fluid} \times \text{normality of alkali})$$

If the sample is 0.200 cc., and if 1.000 cc. of standard acid of 0.200 normality is used and if the alkali used in titration is 0.2 N, the calculation is

$$\text{Gm. N per 100 cc.} = 1.400 - 1.400 \times \text{cc. of titration fluid}$$

In all cases the difference between the blank titration and the titration of the standard acid alone must be subtracted from the gross titration value for the sample.

Accuracy—The limits of the method are given by the titration of standard acid controls. With the Rehberg microburette and measurement of the acid with the Krogh-Keys syringe pipette such controls are readily repeatable within ±0.0002 cc. with 1 N KOH and within ±0.0005 cc. with 0.2 N KOH in the burette. The values correspond with ±0.0028 and ±0.0014 mg. of nitrogen. In the actual determination of nitrogen, however, some errors are introduced in the measurement of the sample, the digestion, and the distillation.

[3] The Rehberg microburette may be obtained from the Macalaster-Bicknell Company, Cambridge, Massachusetts.

The ordinary accuracy of the method may be gaged from (1) the agreement between repeated analyses, and (2) comparison with the standard macroprocedure. A large number of data are available on these two points. All analyses in this laboratory are run in duplicate, so that we have thousands of repeat determinations with the method. In six series taken at random (forty-six sets of results) from the note-books of the past 2 years, the mean discrepancy between duplicates amounted to ±0.00048 cc. in titration or ±0.00060 milliequivalent (the KOH averaged 1.233 N). The greatest discrepancy was 0.0009 cc. or 0.0011 milliequivalent. These values agree with our general experience; we repeat the measurement when a duplicate titration disagrees

TABLE I

Appropriate Quantities and Concentrations for Different Concentrations of Nitrogen or Protein

N concentration as protein*	Sample	H_2SO_4		Concentration of KOH to titrate
		Volume	Concentration	
gm. per 100 cc.	*cc.*	*cc.*	*N*	*N*
0- 0.03	5	1.0	0.02	0.1
0- 0.8	2	1.0	0.2	1.0
0- 9†	0.2	1.0	0.2	1.0
9-18	0.2	2.0	0.2	1.0

* Calculated from protein = 6.25 × nitrogen.

† All ordinary blood serum or plasma.

by as much as 0.0012 cc. and such repetitions are only very rarely necessary.

For more than a year we made a routine practice of running duplicates by the standard macroprocedure on at least every tenth blood sample. No systematic error was found in approximately 150 such comparisons and the agreement of the two methods was always better than the sum of the average errors of the two separate methods. Equally satisfactory results were obtained by eight different technicians in my own laboratories and by other analysts in several other laboratories which have adopted the method.

DISCUSSION

We have found the volumes and concentrations given in Table I cover all ordinary concentrations of nitrogen to be analyzed.

The sensitivity of the method can be increased somewhat by adjusting concentrations and quantities of the reagents more closely to the amount of nitrogen in the sample.

Under ordinary conditions of work, from six to eight determinations, including blanks and controls, may be carried out from start to finish in 2 hours by a single analyst. In a very busy laboratory it is sometimes advantageous to operate several stills; a single analyst can run as many as forty determinations in a day with two stills.

SUMMARY

A micro-Kjeldahl method is described. The method is more rapid than the ordinary macro-Kjeldahl procedure and is not appreciably less accurate. Nitrogen quantities corresponding to that contained in 0.1 to 0.2 cc. of blood serum are very satisfactory.

The method involves measurement of samples and standard acid with syringe pipettes, distillation in a partial vacuum, and titration with a 0.2 cc. capacity microburette.

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MOLECULAR WEIGHT OF EGG ALBUMIN

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Estimates of the molecular weight of egg albumin range from 33,800 (1) to 40,500 (2). This paper deals with the molecular weight of egg albumin as determined from a large number of analyses of tyrosine, tryptophane, and phenylalanine. Analyses of total nitrogen, amide nitrogen, and the known basic amino acids were carried out in an attempt to check the postulate of Bergmann and Niemann (3) that the egg albumin molecule contains 288 amino acids.

EXPERIMENTAL

Egg Albumin—Egg albumin was isolated by the method of Cole (4). After two recrystallizations the protein was dialyzed and electrodialyzed, with the apparatus described by Bernhart, Arnow, and Bratton (5). The protein solution was air-dried in large glass trays protected from dust, and then stored over phosphorus pentoxide. A solution of this dry preparation in distilled water had a pH of 4.75. 98 per cent of a solution of this preparation was coagulated by heat. 97 to 98 per cent of the dried egg albumin was soluble in distilled water. The ash content was 0.014 per cent and the loss in weight upon drying at 105° to constant weight was 1.0 per cent. All analyses are calculated on a moisture- and ash-free basis.

Phenylalanine—Phenylalanine was determined by the Kapeller-Adler (6) procedure.

Tyrosine and Tryptophane—Tyrosine was determined by the method of Folin and Marenzi (7). Tryptophane was determined by Lugg's (8) procedure and by the method of Folin and Marenzi (7). An Eastman No. 61 green filter was placed over the

colorimeter eye-piece during colorimetric comparison of tyrosine. The same filter was used for tryptophane determined by the Lugg method.

Basic Amino Acids—The hexone bases were determined by the method of Block (9).

Total and Amide Nitrogen—Total nitrogen was determined by the Kjeldahl method (10). Amide nitrogen was determined after

TABLE I

Calculation of Molecular Weight of Egg Albumin from Analysis

Amino acid	Method	No. of analyses	Average error of single determination	Average	Determination by other investigators	Bibliographic reference No.	Minimum mol. wt.	No. of moles assumed	Minimal mol. wt.
			per cent	*per cent*	*per cent*				
Tyrosine	Folin and Marenzi	19	0.03	3.89	3.93	(7)	4,650	4	18,600
Phenylalanine	Kapeller-Adler	11	0.06	5.37*	5.07†	(12)	3,100	6	18,600
Tryptophane	Lugg	8	0.03	1.11‡			18,400	1	18,400
"	Folin and Marenzi	16	0.03	1.13	1.16	(7)	18,100	1	18,100
Average									18,400

* Individual analyses (uncorrected for moisture) have been published (11).

† Determined by isolation.

‡ Five of the values were obtained after addition of known amounts of tryptophane to the unknown.

4 hours of hydrolysis with 6 N HCl. Excess HCl was removed by vacuum distillation. Aliquots of a solution of the pasty residue were made alkaline by addition of excess potassium carbonate and the ammonia was aerated into standard acid.

Results

The values obtained by analyses of tyrosine, phenylalanine, and tryptophane are shown in Table I.

From the analytical data obtained, the minimal molecular weight of egg albumin is estimated to be 18,400. Data obtained from physical methods give values for the molecular weight of egg albumin varying from 34,000 (13) to 40,500 (2). The above value of 18,400 multiplied by 2 gives a value of 36,800 for the molecular weight of egg albumin.

TABLE II

Nitrogen of Egg Albumin

Determination	Atoms or molecules per mole (36,800)	Found	Determination by other investigators	Determination by other investigators: Bibliographic reference No.	Calculated	Non-peptide N
		per cent	*per cent*		*per cent*	*atoms per M*
Total N	400	15.27	15.36	(15)	15.27	
Amide "	28	1.07	1.13	(16)	1.07	28
Histidine	4	1.6* 1.7†	1.5	(17)	1.7	8
Arginine	12	5.3‡	5.4	(17)	5.7	36
Lysine	12	4.6§	4.8	(17)	4.8	12
Tryptophane	2	1.1	1.2	(7)	1.1	2
Polysaccharide (5% N, mol. wt. 1200)	1		3.5	(14)	3.3	4
Total						90
						400 − 90 = 310

* Purified by mercury and copper precipitation.

† Not purified by mercury and copper precipitation.

‡ Corrected for solubility of arginine silver (18).

§ Corrected for solubility of lysine phosphotungstate (19).

This value agrees closely with the value (35,700) calculated by Bergmann and Niemann (3). Recently Neuberger (14) isolated 3.5 gm. of a carbohydrate prosthetic group from 100 gm. of recrystallized egg albumin. The isolated polysaccharide contained 5.0 per cent N and had a molecular weight of around 1200.

Bergmann and Niemann did not consider the presence of carbohydrate in their calculations of molecular weight. If it is assumed

that 1 molecule of polysaccharide is present in each egg albumin molecule, as Neuberger's (14) data indicate, consideration of both sets of data leads to an estimation of 36,900 as the molecular weight of egg albumin.

After the molecular weight was estimated, an attempt was made to find the value for the number of amino acids per mole of egg albumin. The following method was used. If the nitrogen atoms known not to be joined in peptide bonds are subtracted from the total number of nitrogen atoms per mole, the difference represents the nitrogen atoms joined in peptide bonds and present at the end of the peptide chain. If the view is accepted that the protein molecule is essentially a peptide chain, then this figure represents the number of amino acids. Nitrogen atoms which are known to exist in the side chains are (1) amide nitrogen, (2) nitrogen of the prosthetic group, and (3) 1 atom of nitrogen for each molecule of lysine and tryptophane, 2 atoms of nitrogen for each molecule of histidine, and 3 nitrogen atoms for each molecule of arginine. The result will not be correct if unknown basic amino acids or nitrogen-containing prosthetic groups are present.

The analytical results and calculations are shown in Table II.

DISCUSSION

With the calculation employed by Bergmann and Niemann (3) to estimate the molecular weight and number of amino acids per mole of protein, an average amino acid residue weight must be assumed unless the protein has been completely analyzed. In the case of egg albumin in which only about two-thirds of the total amino acids has been identified (20) there is no way of finding out the average amino acid residue weight by direct chemical means (21).

In this paper in the calculation of molecular weight from chemical composition the assumption of an average amino acid residue weight is avoided and analyses of tyrosine, tryptophane, and phenylalanine are depended upon solely. In the calculation of the number of amino acids per mole of egg albumin the assumption of an average amino acid residue weight is likewise avoided, but the result obtained rests upon the assumption that no unknown basic amino acids or nitrogen-containing prosthetic groups are present in the egg albumin molecule.

Both methods of calculation give closely agreeing values for

the molecular weight. The number of amino acids per mole calculated by the present method (310) approaches closely the value of 288 calculated by Bergmann and Niemann.

The agreement obtained may be considered as evidence of the correctness of the assumptions made by Bergmann and Niemann in regard to the chemistry of egg albumin.

SUMMARY

1. Analyses of the tyrosine, tryptophane, and phenylalanine content indicate a minimal molecular weight of 18,400 for electrodialyzed, moisture-free egg albumin.

2. Calculations indicate the presence of 310 amino acids per mole of egg albumin. This result rests on the assumption that no unknown basic amino acids or nitrogen-containing prosthetic groups are present in the egg albumin molecule.

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APPARATUS FOR THE CONTINUOUS DRYING AND EXTRACTION OF BIOLOGICAL MATERIALS. APPLICATION TO THE EXTRACTION OF THE NEUTRAL FAT FRACTION OF FECES

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The extraction of liquid or moist biological materials almost invariably involves preliminary drying of the sample. This is usually accomplished by heating the finely divided specimen on a steam bath or in a drying oven at 105–110°. The length of time necessary for complete dehydration depends upon the surface exposed, the temperature of drying, and the circulation of air above the material. One method of insuring (1) complete desiccation involves the addition of a volatile water-soluble solvent after the sample has been dried sufficiently to be powdered, and then heating to drive off the solvent. Final traces of moisture are then removed after the sample has stood for several days in a vacuum desiccator.

It is quite apparent that these procedures are open to several serious objections. In the first place, the analyst can never be certain that his sample is absolutely free of water. Water and steam pockets are frequently encountered. Prolonged heating may cause decomposition. Volatile solvents added to aid drying usually extract fats which deposit on the walls of the drying vessel and the final product is not truly homogeneous in composition. As a consequence of the many precautions which are thus taken, the process of drying involves a considerable expenditure of time. Days, and sometimes weeks, are required for an analysis involving a preliminary drying. Then, too, analyses of very fatty substances are a problem in themselves. A gummy product results, one which contains water bound in such a fashion that it

cannot be removed. The substance defies pulverization and causes the analyst great inconvenience.

However, the most serious objection that can be leveled at any drying process that involves prolonged heating at temperatures of 100° or higher is that of the decomposition engendered. Easily oxidizable materials combine with the oxygen of the air at these temperatures. Some (2) prevent this by heating *in vacuo*. The elevated temperature, nevertheless, does not prevent substances other than water from escaping. Volatile substances and steam-distillable materials are lost. Some fatty acids, important constituents in lipid fractionation, are believed to be lost in this fashion. Obviously, the dry weight then is not a true picture of the amount of water contained in the sample analyzed.

It was with these facts in mind that the present study was undertaken. A drying procedure was sought which would remove only water, involve a minimum of time, and give complete desiccation. Since the next step in the analysis of the dried material usually involves an extraction, an apparatus was sought which would perform both tasks; namely, drying and extraction.

The Dean and Stark (3) moisture distillation apparatus was first tried, with a volatile liquid. Complete drying was obtained in 3 to 4 hours by suspending 100 gm. of wet feces in boiling benzene. The dried and powdered stool, when compared with a sample of the same specimen dried on a steam bath, offered a sharp contrast in so far as physical appearance was concerned. The latter consisted of a dark brown to black powder, while the former was light tan in color. It is quite apparent that some drastic changes must have occurred in the feces which had been dried on the steam bath.

This apparatus, however, was not completely satisfactory. As previously pointed out, during drying in an organic solvent fats are extracted which deposit on the walls of the drying vessel after removal of the solvent. Furthermore, an apparatus was sought which would continuously extract as well as dry the material under investigation.

A special apparatus was designed and constructed with these ends in view. Fig. 1 is a diagram of the apparatus. The wet sample is weighed into a weighed extraction thimble, previously extracted and dried, and placed in compartment *B*. The solvent

is then introduced through the mouth of *C*, the apparatus having been set up and connected, with the exception of the condenser *D*. Enough solvent is admitted barely to reach the level of the side arm through which siphoning occurs. The condenser is then connected. Water baths are placed under *A* and *B* and maintained at a temperature sufficient to create a vigorous ebullition

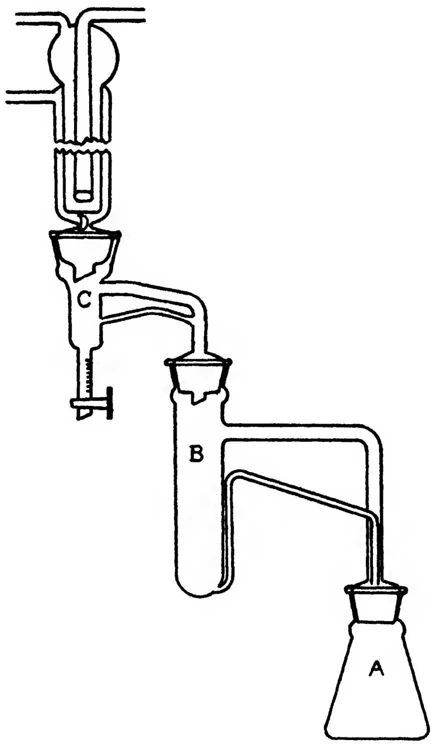

FIG. 1. Apparatus for continuous drying and extraction of biological materials.

of the solvent. Though no solvent is placed in flask *A*, a hot water bath must surround it to volatilize any solvent which drips into it. The water contained in the stool has an appreciable vapor pressure at the boiling point of the solvent chosen (60–80°) to distil over to *C* together with the solvent. Condensation of the vapors permits the water to settle at the bottom of *C*, owing to its

greater specific gravity. The water content can then be read directly. Drying usually involves 1 to 2 hours.

At the end of this time, the water bath surrounding *B* is cooled to a temperature just below the boiling point of the solvent. Extraction now occurs. This is continued as long as is necessary. When extraction is complete, the thimble and its contents are dried at 110° for 2 hours and weighed. The solvent in flask *A* is removed by distillation in the same apparatus, being collected by opening the stop-cock at *C*. The last traces of solvent are removed by applying a slight vacuum to the flask. The weight of the residue in flask *A* added to the weight of the solid material in the extraction thimble represents the dry weight of the substance.

This procedure has several advantages over those generally followed. A true dry weight is obtained, volatile materials being extracted by the solvent and caught in flask *A*. Oxidation is prevented by avoiding high temperatures and an atmosphere of oxygen, the vapors of the solvent having displaced the air. Consequently, higher dry weights should be expected. The water content can be read directly at *C*, an advantage possessed by no other method. The drying time is cut considerably and the substance is thoroughly dried. No water pockets can form here because the boiling solvent seeps into the most inaccessible recesses. The extraction is more thorough than in usual extractions in an ordinary Soxhlet extraction apparatus. The use of the latter has long been recognized as an efficient and convenient process for the extraction of dried materials. The apparatus in Fig. 1 affords a means of performing a hot extraction in contradistinction to the cold or at best warm extraction of the Soxhlet apparatus. It also permits of the convenient removal and recovery of the solvent in flask *A*, making the extraction and drying process very inexpensive.

The apparatus itself can be put to many uses other than that just described. This is due to the fact that all connections are interchangeable. It can, therefore, when *A* is connected with the condenser, be used for ordinary refluxing. Without *C*, it can serve as a Soxhlet apparatus. Without *B*, it can serve as a drying apparatus similar to that of Dean and Stark. It is evident, therefore, that such an apparatus has many and varied uses.

Extractions of drugs, as sulfanilamide and its derivatives, poisons in toxicological work, and lipids are but a few of its many applications. As an example, a study of the extraction of the neutral fat fraction (consisting of neutral fat, fatty acids, and unsaponifiable substances) from feces was undertaken. The importance of this determination clinically was one of the chief factors influencing this selection. There is a vast literature on this subject, many methods being in use. One widely used is that of Holt, Courtney, and Fales (4), which is essentially an adaptation of the Rose-Gottlieb method for the analysis of milk powders. Subsequent workers (1, 5) have altered the procedure in one way or another with a view to improving its accuracy, but it still remains troublesome and time-consuming. One of the latest modifications of this procedure is that of Tidwell and Holt (2), by which some of its difficulties are avoided. Dried stools are extracted four times with a mixture of ethyl and petroleum ethers, centrifuging being employed to prevent the tendency of emulsions to be formed during the extractions. While this method gives excellent results on the recovery of fats, fatty acids, and unsaponifiable material added to stools, it is doubtful whether all the fat is actually extracted. Other experimenters (5, 6) have used ethyl ether or petroleum ether in a Soxhlet extraction apparatus for the same purpose. Long periods of extraction, however, are necessary. Warth (6) found that the material extracted by petroleum ether from feces in 16 hours amounted to 80 per cent of that extracted in 108 hours. Then, too, it is a disputed question whether the analysis of feces dried on a steam bath is the best procedure to follow. Although there is an extensive lipid literature based on this way of drying, Fowweather (7) and others point out that it is inaccurate. Fowweather claims more complete extraction of fat from wet stools, while Folin and Wentworth (5) point out that drying on a steam bath causes the formation of soaps from the fatty acids and volatilizes the more volatile fatty acids.

It seemed desirable therefore to apply our apparatus to the problem. Wet feces could be used, thus insuring a better extraction and a higher fatty acid fraction. Higher fat results should be obtained than those gotten by procedures involving but a few extractions in a separatory funnel or centrifuge tube,

because of the continued extraction over a longer period of time which an extraction apparatus affords.

A solvent was sought which should have the following characteristics. (1) It should have a boiling point much lower than that of water but high enough to remove it from the feces in a reasonable length of time. A temperature of 60–70° was thought optimum. (2) It must be immiscible with and dissolve a minimum amount of water. (3) Its density must be sufficiently lower than that of water to permit of rapid settling of the latter without the formation of troublesome emulsions. (4) It must be an excellent fat solvent, yet extract a minimum of other materials, especially soaps. (5) It should be inexpensive and readily available on the market.

A number of solvents were tried but only two proved at all satisfactory. The first, benzene, was discarded because of its tendency to form emulsions with water and because its boiling point was thought too high. A search of the literature failed to reveal the use of the second in any fat analysis of fecal material. This compound is isopropyl ether, a liquid having a density of 0.7258 (20/4) and a boiling point of 67.5°. It is an excellent fat solvent, dissolves very little water, does not form emulsions with water, and is rather cheap and readily available. In fact, as will be shown later, it is far superior to the more common fat solvents. Furthermore, it was found that the time required for complete extraction of fat was lessened materially, complete extraction being secured in about 10 hours.

With the suggested procedure it is believed that a more complete extraction in a shorter period of time, requiring less attention on the part of the analyst, and giving a truer picture of the distribution of the components of the neutral fat fraction of feces, is obtained.

Apparatus—The apparatus in Fig. 1 consists of four parts labeled *A*, *B*, *C*, and *D*. All connections are interchangeable ground glass connections (Pyrex No. 29/42), whose top diameter measures 29.2 mm. and whose bottom diameter is 25.0 mm. The entire apparatus is made of Pyrex glass.

A consists of an ordinary 125 ml. Erlenmeyer flask to which has been sealed the female part of the ground glass joint.

B is the extraction and drying chamber. It is 20 cm. in length

and its outside diameter is 3.44 cm. The outside diameter of the upper side arm is 1.29 cm. This side arm makes a right angle bend 6.7 cm. from the main tube and extends 17.5 cm. from the bend. The upper side arm is 17 cm., and the lower rises 10 cm., from the bottom of the tube. The outside diameter of the inner side arm is 0.53 cm., the bottom of which is 1 cm. below that of the main tube. The main tube and the side arm are both sealed to the male parts of the ground glass joints.

C is the moisture trap. The advantage of this specially designed trap is that once the water is distilled up into the condenser and drops into the trap, it must stay there. With the usual type of Dean and Stark trap the drops of water form beads on the surface of the solvent or an emulsion in the trap and are carried back into the flask. This modification does not permit the water to return to the flask because the flow through the side tube is not strong enough to carry the falling drops up into it. The tube is 20.8 cm. in length from the top of the male ground glass joint to the tip of the stop-cock. Below the joint the tube measures 1.6 cm. in outside diameter and tapers into a tube of 5 cm. diameter (outside), graduated in tenths of a ml. and of 5 ml. capacity. The larger side arm has an outside diameter of 1.6 cm. and slopes very slightly to a right angle bend about 6 to 7 cm. from the main compartment. The length of the side arm from this bend to the bottom of the female joint is 18.7 cm. The smaller side arm has an outside diameter of 0.53 cm. and is sealed to the tube at a height of 1 to 2 cm. above the graduated section. It has a slight bend in it and is sealed to the side arm at a slightly greater height than in the main tube. *D* is a standard Hopkins condenser whose cooling length is 20 cm. The use of a Hopkins condenser in place of a Liebig condenser prevents the condensation of water on the outside of the condenser jacket.

Method

3 to 5 gm. of moist feces are weighed into an extraction thimble (Whatman, inside length 50 mm., inside diameter 19 mm.) which has been previously extracted for 2 hours with isopropyl ether in the apparatus (Fig. 1) and then dried for a similar length of time in an oven at 100–110°. These thimbles can be kept indefinitely in a vacuum desiccator after this treatment. Weighings are best

performed in weighing bottles, owing to the tendency of the thimbles to take up water and of the stool to lose water by evaporation. It is imperative that the stool selected be a representative sample. This can be accomplished by adequate mixing in a mortar previous to weighing.

The thimble is now placed in part *B* and the apparatus connected, with the exception of the condenser. Isopropyl ether (redistilled over sodium, boiling point 66–69°) is then admitted through the mouth of *C* until sufficient solvent has been added barely to reach the level of the side arm through which siphoning occurs. The condenser is then connected and the water baths under *A* and *B* heated until the isopropyl ether boils vigorously. This is continued for about 1½ hours. At the end of this time, the water bath under *B* is cooled to about 60° and maintained at this temperature for the remainder of the extraction, 10 hours. At the end of this time, the volume of water at *C* is noted and discarded, the water bath under *B* is heated to boiling, and the solvent distilled and collected at *C*. Flask *A*, whose weight is known, is now removed from the apparatus and the last traces of solvent contained in it evaporated by applying a slight vacuum. When cool, it is weighed. The difference in weight represents the amount of isopropyl ether-extractable material. This consists almost entirely of the neutral fat fraction and is purified by solution in petroleum ether (boiling point 35–60°) and filtration into a weighed 100 or 150 ml. beaker. Owing to the tendency of lipids to be absorbed by filter paper and to the difficulty encountered in washing it free, a special filtration procedure is suggested at this stage. The petroleum ether is decanted into a 50 ml. centrifuge tube, centrifuged at 3000 R.P.M. for 5 minutes, and decanted through a funnel loosely stoppered with a small plug of cotton. Three or four transfers of about 30 cc. each are usually found adequate. The petroleum ether is evaporated on a water bath to a small volume. The last traces are removed *in vacuo*. The weight of the residue in the beaker represents the amount of neutral fat, fatty acids, and unsaponifiable material contained in the original sample.

Fatty acids can be estimated by dissolving this residue in 20 cc. of 95 per cent ethyl alcohol and titrating with aqueous 0.1 N sodium hydroxide near the boiling point of alcohol. Phenol-

phthalein is used as indicator. 1 drop of a 1 per cent alcoholic solution is added. A blank should be run on the alcohol to determine the amount of alkali necessary to neutralize any acids present. 1 ml. of 0.1 N alkali is assumed to neutralize 28.4 mg. of fatty acid (5).

The dry weight of the stool can be determined by removing the thimble from the apparatus and drying it at 110° for 2 hours. Cool in a desiccator and weigh. The weight of its contents added to the weight of the isopropyl ether-extracted material represents the dry weight of the stool.

Calculation—

Dry weight = weight of dried residue in extraction thimble + weight of isopropyl ether-extracted material in flask *A*

$$\% \text{ solids} = (\text{dry weight})/(\text{wet weight}) \times 100$$

$$\% \text{ water} = \frac{\text{weight of water (volume may be used)}}{\text{weight of wet stool}} \times 100$$

$$\% \text{ neutral fat fraction (dry weight)} = \frac{\text{weight of fraction}}{\text{dry weight}} \times 100$$

$$\% \text{ `` `` `` (wet ``)} = \frac{\text{weight of fraction}}{\text{wet weight}} \times 100$$

Weight of fatty acids (in mg.) = ((titration of unknown) − blank) × 28.4

$$\% \text{ fatty acids (dry weight)} = \frac{\text{weight of fatty acids}}{\text{dry weight}} \times 100$$

% neutral fat and unsaponifiable = % neutral fat fraction − % fatty acids

EXPERIMENTAL AND DISCUSSION

In Table I are listed the results of an experiment comparing the ability of several solvents to extract the neutral fat fraction from feces previously dried in boiling benzene. In the case of Solvents 1 to 7, 500 mg. of dried feces were extracted eight times with 10 ml. portions of the solvent. The solvent was then evaporated and the residue weighed. The residue was dissolved in petroleum ether and filtered, according to the procedure described under "Method," and transferred quantitatively to another weighed beaker. After the evaporation of the petroleum ether, the beaker was reweighed.

In the case of Solvent 8, the stool was previously boiled with 1 ml. of 95 per cent alcohol until all the alcohol was evaporated and then extracted with petroleum ether. This was done in the hope

that prior treatment with boiling alcohol might make the fecal fats more easily extractable. In Solvent 9, the stool was extracted with petroleum ether in an ordinary Soxhlet extraction apparatus for 8 hours. The higher per cent of extractable material in this case was probably due to the fact that some particles of the finely powdered stool were carried over mechanically to the weighed flask. That this was probably the case is shown by the loss in weight after re-solution in petroleum ether. It is to be noted that isopropyl ether extracts more lipids, *i.e.* petroleum ether-soluble material, than any of the other solvents. The results in Table I as in others to be presented are all averages of duplicate analyses.

TABLE I

Petroleum Ether-Soluble Material in Fecal Extracts of Various Fat Solvents

Solvent	Per cent extractable material	Per cent petroleum ether-extractable material
1. Petroleum ether	20.0	19.8
2. Ethyl ether	21.2	20.8
3. Benzene	20.9	20.3
4. Bloor's reagent (alcohol-ether 3:1)	19.6	14.6
5. Acetone	13.4	13.0
6. Isopropyl ether	37.3	27.4
7. High boiling petroleum ether, b.p. 60–67°.	19.1	17.7
8. Prior treatment with alcohol followed by petroleum ether extraction	18.1	18.1
9. Extraction with petroleum ether in Soxhlet apparatus for 8 hrs.	22.6	20.3

In our later work, extraction with petroleum ether in a Soxhlet extraction apparatus over a period of several hours was chosen as a reference method for comparison with our own procedure. This can be justified on the grounds that this solvent extracts as much fat as most other solvents tested.

Dry weights obtained by drying on a steam bath are compared with dry weights obtained by suspending the stool in boiling benzene, in Table II, and in boiling isopropyl ether, in Table III. As has been predicted, owing to decomposition and volatilization of volatile constituents, higher per cent solids are gotten by drying in an organic solvent than by drying at elevated temperatures in the presence of air.

In Table III, the authors' method of extraction is compared with another method of extraction. Feces, dried on a steam bath were extracted with petroleum ether for 5 hours in a Soxhlet apparatus. In the authors' procedure, the stool was dried in boiling isopropyl ether for 1½ hours, then extracted at 60° for 3½ hours with the same solvent. A great deal more fat, richer in fatty acids, is extracted by the proposed procedure than by the

TABLE II

Per Cent of Solids in Feces

Sample	Per cent solids, benzene-dried	Per cent solids, dried on steam bath
A	29.5	27.7
B	26.8	26.4
C	29.5	28.7

TABLE III

Comparative Analysis of Solids, Neutral Fats, and Free Fatty Acids in Feces

Sample No.	Per cent dry weight		Per cent neutral fat fraction		Per cent fatty acids		Fatty acids, per cent of fecal fat	
	Authors' method	Dried on steam bath	Authors' method	Petroleum ether extraction of dried feces	Authors' method	Petroleum ether-extracted	Authors' method	Petroleum ether extraction
1	26.9	25.6	19.8	17.9	11.7	3.9	59	22
2	34.8	33.1	20.5	13.1	10.6	4.5	52	34
3	33.5	30.9	15.9	9.6	9.0	2.5	57	26
4	17.3	16.6	16.8	8.9	10.1	4.1	60	46
5	29.7	27.2	19.9	17.9	12.1	3.6	61	20
6	29.4	25.8	21.7	17.9	13.7	2.6	63	15

other method. No doubt the volatile fatty acids which are lost in drying on the steam bath are an important factor in this increase. Another reason may be found in the work of Folin and Wentworth (5) who suggest that soap formation is influenced by the application of heat and the presence of hydrogen sulfide, carbon dioxide, ammonia (substances found in air and which may be present during drying), calcium, and magnesium. The soaps are probably formed at the expense of the fatty acid fraction. This

would account for the low percentages of fatty acids found in feces which had been dried in air at high temperatures.

The authors' method is compared with that of Tidwell and Holt (2) in Table IV. Wet stools were used in their procedure for the sake of maintaining comparable conditions. In their procedure the feces are suspended in boiling 50 per cent alcohol, and extracted four times with a mixture of ethyl and petroleum ethers. This was done, but many more extractions were performed, thirty in all. The combined extracts were evaporated and the residue

TABLE IV

Comparison of Present Method of Fecal Analysis with That of Tidwell and Holt

Per cent neutral fat fraction		Per cent fatty acids		Per cent fatty acids of fecal fat	
Authors' method	Tidwell and Holt	Authors' method	Tidwell and Holt	Authors' method	Tidwell and Holt
7.50	3.63	4.02	1.16	57	44

TABLE V

Time Necessary for Complete Extraction of Fecal Fat

Time	Neutral fat fraction extracted	Completeness of extraction	Neutral fat fraction extracted	Completeness of extraction
hrs.	*per cent*	*per cent*	*per cent*	*per cent*
3	22.3	91.4	21.0	91.7
10	24.0	98.4	22.6	98.6
17	24.3	99.6	22.8	99.6
24	24.4	100	22.9	100
36	24.4	100	22.9	100

purified by dissolving it in petroleum ether and filtering. The results in Table IV are based on the weight of wet stool. Higher fat values are obtained in the authors' method, in which extraction was continued for 10 hours after the 2 hour period of drying.

The physical appearance of the fat extracted by the authors' procedure offers a sharp contrast with that extracted by other methods. The first is usually colored a light yellow or yellowish green, is invariably crystalline, and easily soluble in warm 95 per cent alcohol. The titration of the fatty acids here is comparatively simple, the end-point being fairly sharp. Not so in the case of

other procedures tried. Here the fat is a dark brown amorphous mass, most of which is insoluble in alcohol. Consequently, the fatty acid titration is not as simple. The end-point is difficult to perceive owing to the masking of the red color of phenolphthalein in alkaline solution. A true titration is difficult to obtain because some fatty acids are encased in the gummy masses which defy solution. Vigorous stirring is necessary to overcome this.

The results of a study of the time necessary for complete extraction of the fat are given in Table V. Complete extraction is secured in 24 hours. However, since almost complete extraction is obtained in 10 hours, extraction for this length of time is recommended. The small error in this case, which amounts to about 1.5 per cent, and the greater convenience justify this change.

SUMMARY

1. An apparatus is described which is capable of drying biological materials, such as feces, tissues, organs, bones, blood, and other body fluids, and extracting organic substances such as fats, drugs, and poisons.

2. With this apparatus, a study has been made of the neutral fat fraction of feces. A method has been proposed and compared with others widely used.

3. A new solvent for the extraction of fat from feces has been introduced which has proved more efficient than other common fat solvents.

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THE ACTIVATION ENERGY OF UREA HYDROLYSIS CATALYZED BY SOY BEAN UREASE

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The rate of most chemical reactions increases exponentially with temperature in accordance with the Arrhenius equation. The older literature on enzyme-catalyzed reactions (for references *cf.* Haldane (1930), Tauber (1937)) indicates that these do not conform, since the activation energy decreases with rise in temperature instead of being invariant. More recent studies do not confirm this general statement, however, but indicate that the reaction velocity of certain enzyme systems increases with temperature in conformity with the Arrhenius equation up to the inactivation temperature of the enzyme (Bodansky, 1939; Craig, 1936; Crozier, 1924; Gould and Sizer, 1938; Hadidian and Hoagland, 1939; Sizer, 1937, 1938, 1939). Bodansky (1937) has emphasized the fact that in much of the earlier work little attention was paid to pH control, and velocity constants were often improperly computed.

A comparison of the activation energies of catalytic systems in which the enzyme has been obtained from different species can yield valuable information concerning the biochemical relationships of enzymes. Sizer (1937) reported an activation energy of 13,000 calories per gm. mole for sucrose hydrolysis by malt invertase as compared with 11,000 calories for yeast invertase (1938). Bodansky (1939), on the other hand, obtained a value of 9940 calories for the hydrolysis of sodium β-glycerophosphate by either human or cat bone phosphatase. Activation energies of either 8700 or 11,700 calories were reported (Sizer, 1939) for urea hydrol-

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ysis by crude or crystalline jack bean urease. With this figure may be compared the calculation of von Euler (1920) of 20,800 calories, later corrected to 12,000 (1922), from the data of Van Slyke and Cullen (1914) on soy bean urease. The latter figure was checked by Sizer (1939) who obtained a value of 11,700 calories.

In this study the hydrolysis of urea catalyzed by soy bean urease has been investigated as a function of temperature in order to compare the activation energy with those of the jack bean urease system. If jack and soy bean ureases are comparable, it might be possible to obtain activation energies of both 8700 and 11,700 calories under the proper conditions for soy as well as jack bean urease.

EXPERIMENTAL

The urease was purified from either Arlco[1] or Cellu[2] soy bean flour. Enzyme solutions were prepared according to the method of Van Slyke and Cullen (1914) by suspending the flour in water and separating the urease solution from the insoluble material by filtration or centrifugation. In addition to this aqueous solution of urease a large number of more highly purified preparations were made. These were prepared by successive precipitations of the urease by various concentrations of acetone, alcohol, and ammonium sulfate. After each precipitation the enzyme was dissolved in water. In some cases the final urease solution was dialyzed free of non-colloidal material. Besides the studies which were made on the urease purified to various degrees from two different soy bean flours, tests were also performed in which unmanipulated and unmodified urease as present in the bean (yellow variety) was used. The temperature kinetics of unextracted urease in the soy bean were compared with jack bean urease in a similar state.

A stock solution was prepared which contained 3 per cent urea, 5.4 per cent Na_2HPO_4, and 4.25 per cent KH_2PO_4. The phosphate buffered the digest to pH 7.0 (optimum pH for urease activity) and kept the alkalinity produced by the liberated NH_3 from increasing by more than 0.1 pH unit while the reaction was being

[1] Arlington Chemical Company, Yonkers, New York.

[2] Chicago Dietetic Supply House, Inc., Chicago.

studied. To 2 ml. of urea-phosphate solution were added 1 ml. of enzyme solution and 1 ml. of stabilizer which was usually an oxidizing or a reducing solution. The stabilizer was added in view of the fact that the urease molecule is unstable and can be readily activated or inactivated by the addition of reducing or oxidizing agents (Hellerman, 1937). The activation energy of the jack bean urease-urea system is 11,700 calories when oxidizing agents are present, but is 8700 calories in the presence of neutral or reducing agents (Sizer, 1939).

During the course of urea hydrolysis the liberated NH_3 dissolves in the solution, while the CO_2 is evolved and can be measured manometrically (Van Slyke, 1927; Krebs and Henseleit, 1932). Hydrolysis follows the same apparent course whether studied by measurement of NH_3 production colorimetrically after nesslerization or CO_2 evolution manometrically with the Barcroft differential manometer (Sizer, 1939).

The 4 ml. of reaction mixture were placed in one cup of the manometer and 4 ml. of water in the control cup. From 2 to 3 minutes adaptation to the temperature of the water bath were allowed before the stop-cocks were closed. From ten to fifteen manometer readings were taken at each temperature during the time required for the evolution of 100 to 500 c.mm. of gas. The temperature of the water bath was controlled to $\pm 0.05°$. A pressure change of 1 mm. on the manometer was found to correspond to a change in volume of 2.6 c.mm. Since the number of molecules in a unit volume of gas at a given pressure varies with the temperature, it was necessary to convert the gas volumes to the standard temperature of 0° to make them comparable.

Results

In Fig. 1 is plotted CO_2 evolution as a function of time for a solution containing 2 ml. of urea-phosphate, 1 ml. of water, and 1 ml. of urease (saturated $(NH_4)_2SO_4$ precipitate of a 30 per cent aqueous extract of Cellu soy bean flour). From the figure it is apparent that the reaction follows a linear course during the first phase of the hydrolysis at all temperatures between 0.2–50°. Rates of hydrolysis were calculated from the slopes of the straight lines drawn through the plotted points and expressed as ml. of CO_2 evolved per minute. This proved to be an accurate method

of determining rate, since errors of separate readings are largely eliminated.

While the data of Fig. 1 are typical, there were occasional enzyme preparations which were so unstable that the plotted points fell off from a straight line after only 0.2 to 0.3 ml. of CO_2 had been evolved. In a few experiments a brief lag occurred at the start of the reaction. In all cases, however, it was possible to calculate rate of hydrolysis from the linear portion of the curve.

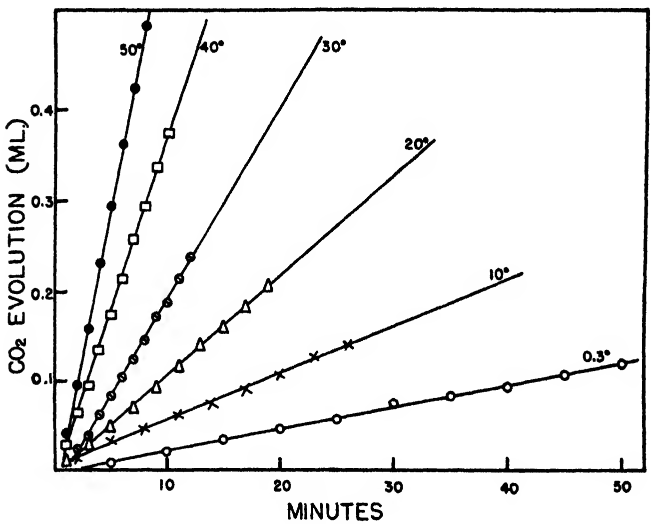

FIG. 1. Hydrolysis (as measured by ml. of CO_2 evolved) of 1.5 per cent urea (in phosphate buffer, pH 7.0) by soy bean urease is plotted as a function of elapsed time in minutes for several different temperatures. The reaction follows a linear course at all temperatures.

In Fig. 2 log rate of hydrolysis is plotted against the reciprocal of the absolute temperature. Since the plotted points are best fitted by a straight line, it is clear that the data are in accord with the Arrhenius equation

$$\mu = \frac{2.303R \log (k_2/k_1)}{1/T_1 - 1/T_2}$$

where μ is the energy of activation in calories per gm. mole, R the gas constant, and k_1 and k_2 the rates at the respective absolute

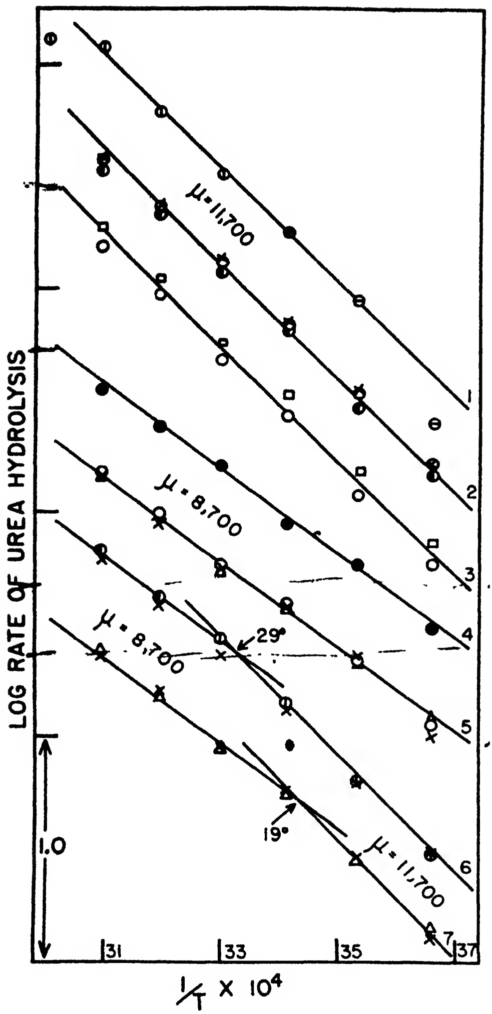

FIG. 2. Log rate of urea hydrolysis by soy bean urease plotted against the reciprocal of absolute temperature. The distribution of the curves along the ordinate is arbitrary. Data of Van Slyke and Cullen on *hydrolysis as measured by NH_3 formation,* Curve 1; ⦶, dilute urease solution used; ⊖, concentrated urease solution used. *Hydrolysis as measured by CO_2 evolution,* Curves 2 to 7. For details of each urease preparation see the text. In each experiment the digest contained 2 ml. of urea-phosphate, 1 ml. of urease, and 1 ml. of one of the following: ○, H_2O; ×, 0.2 M $K_3Fe(CN)_6$; ◐, 0.2 M $K_4Fe(CN)_6$; □, 0.14 M sulfite; ●, 0.2 M $Na_2S_2O_3$; △, 0.2 M KCN.

temperatures T_1 and T_2. The distribution of the curves along the ordinate is purely arbitrary. The slope of Curves 1, 2, and 3 corresponds to an activation energy of 11,700 calories. In Curve 1 are presented the data of Van Slyke and Cullen (1914) who followed hydrolysis by distilling off and titrating the liberated NH_3. The velocity constants of the data for concentrated urease have been adjusted so that the 20° point coincides with that for the dilute urease. The 60° point is doubtless low because of temperature inactivation of the enzyme which occurs between 40–60°, depending upon a variety of factors. In Curve 2 are presented the data of Fig. 1, as well as those procured when 1 ml. of 0.2 M $K_3Fe(CN)_6$ or 1 ml. of 0.2 M $K_4Fe(CN)_6$ was used as stabilizer instead of 1 ml. of H_2O. For Curve 3 the Arlco soy bean urease was purified from the aqueous extract by successive precipitation with saturated $(NH_4)_2SO_4$, 50 per cent alcohol, and 50 per cent acetone. Water or a 0.14 M mixture of Na_2SO_3 and $NaHSO_3$ (pH 7.0) was used as stabilizer.

Strikingly different results are presented in Curves 4 and 5 of Fig. 2 where the data fit straight lines with a corresponding activation energy of 8700 calories. For the data of Curve 4 the urease was a 50 per cent acetone precipitate of a 25 per cent aqueous suspension of Arlco flour; 0.2 M $Na_2S_2O_3$ was the stabilizer. For Curve 5 the urease was purified by successive precipitation of a 10 per cent acetone extract of Arlco flour by 0.5 saturation with $(NH_4)_2SO_4$, 50 per cent acetone, 0.5 saturated $(NH_4)_2SO_4$, and 40 per cent alcohol. The stabilizers were H_2O, 0.2 M $K_3Fe(CN)_6$, and 0.2 M KCN.

Two straight lines intersecting at a critical temperature best fit the plotted points of Curves 6 and 7 of Fig. 2. The corresponding activation energies are 11,700 calories below and 8700 above the critical temperature. For Curve 6 the urease was the same as for Curve 3, but the stabilizers were 0.2 M $K_3Fe(CN)_6$ and 0.2 M $K_4Fe(CN)_6$. For Curve 7 the urease was a 0.5 saturated $(NH_4)_2SO_4$ precipitate of a 30 per cent suspension of Cellu flour. The stabilizers were 0.2 M $K_3Fe(CN)_6$ and 0.2 M KCN.

In a small number of experiments an activation energy of 7000 calories was obtained over the whole temperature range. It is not known whether this represents a new value for the familiar urease-urea system, or whether this figure is indicative of a differ-

ent urease enzyme which is sometimes present in soy beans. With regard to this it is interesting to note that the activation energy of 7000 calories was never obtained with Cellu soy meal, and never with Arlco soy bean urease after it had been highly purified.

From Fig. 2 as well as from 67 additional temperature studies not shown in the figure, it is apparent that the activation energies for the soy bean urease-urea system are 11,700 or 8700 calories over the whole temperature range, or 11,700 below and 8700 above a critical temperature. It appears that the particular value obtained depends upon the following factors: (1) The source of the soy bean flour. One encounters μ = 11,700 calories more frequently with Cellu than with Arlco flour. (2) The method and degree of purification of the urease. Curves 3 and 5 (where water was the stabilizer) are characterized by respective μ values of 11,700 and 8700 calories; yet the only difference in the two series of experiments was the manner in which the urease was purified. (3) The stabilizer added to the reaction mixture. Data for curves 3 and 6 were procured simultaneously, the only difference being the stabilizer employed; yet the temperature effects are very different in the two cases.

Since either of two activation energies can be obtained for the soy bean urease-urea system, it is of interest to learn which of these two values characterizes the urease as it naturally occurs in the bean, where there is no possibility of the urease becoming modified by milling and extraction procedures. For this purpose the protective seed coat was removed from a single bean, the two halves (cotyledons) separated and soaked overnight in either 0.4 M $K_3Fe(CN)_6$, or 25 per cent acetone, or water saturated with toluene. These inhibitors suppressed respiration so that volume changes in the flask were due only to CO_2 liberated from urea. Each bean was placed in a separate Barcroft flask to which were added 2 ml. of urea-phosphate and 2 ml. of the respective inhibitor. After each temperature run the digest was discarded and the bean rinsed three times with water before being used again.

The CO_2 evolution was a linear function of time, just as was the case for hydrolysis by the various urease solutions prepared from the flour. In an Arrhenius plot of the data (Fig. 3) the points fall along a straight line which has a slope corresponding to μ = 11,700

calories, when water saturated with toluene is the stabilizer (Curve 1), and to $\mu = 8700$ calories when 12.5 per cent acetone or 0.2 M $K_3Fe(CN)_6$ is present (Curve 2). In Curve 3 are presented the data for the same experiment with the same inhibitors, but with one-fourth jack bean (one-half cotyledon) substituted for one soy

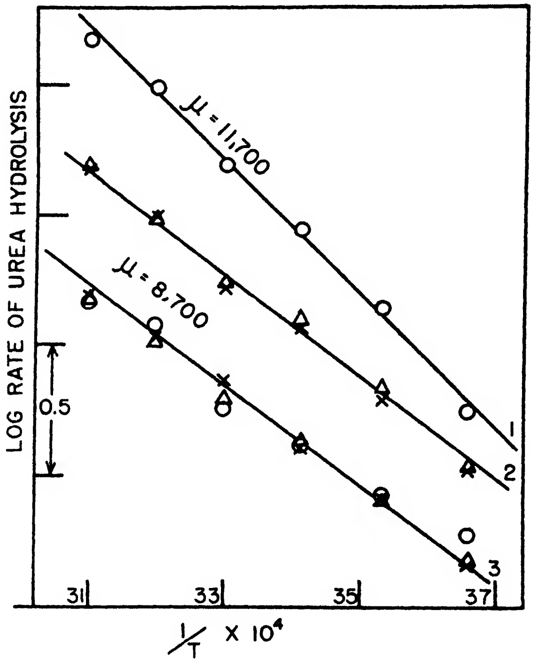

FIG. 3. Log rate of CO_2 evolution from urea catalyzed by urease not extracted from the bean. The Barcroft flasks contained 2 ml. of urea-phosphate plus 2 ml. of one of the following: O, water saturated with toluene; ×, 25 per cent acetone; △, 0.4 M $K_3Fe(CN)_6$. Curves 1 and 2, one soy bean added to digest; Curve 3, one-fourth jack bean added to digest.

bean. Scrutiny of the figure indicates that the data conform to the Arrhenius equation where $\mu = 8700$ calories.

DISCUSSION

Duplicate experiments in some cases were performed at all six temperatures. With different stabilizers two, three, or four separate studies were made on a single enzyme preparation at each

temperature. In duplicate experiments the manometer readings did not differ from the average by more than 5 per cent. The straight lines drawn through the plotted points were fitted by "eye," a method which checks that of the "least squares" within about 2 per cent (Hoagland, 1936). The activation energies are accurate to about ±200 calories.

It appears significant that the activation mechanism for the urease system from two different genera of legumes, *Canavalia ensiformis* and *Glycine hispida*, is essentially identical for the two enzymes. In the case of crystalline jack bean urease an activation energy of 8700 calories was associated with neutral or reducing agents and μ = 11,700 calories with oxidizing agents present in the digest. No such correlation between activation energy and oxidation-reduction potential is apparent for crude soy bean urease, however, in which other factors complicate the situation. For both enzymes a shift in activation energy could be elicited by modifying the composition of the digest. Under certain conditions with both enzymes two activation energies characterize the system with μ = 11,700 calories below and μ = 8700 calories above the critical transition temperature. Although enzymes from different species may not be immunologically identical (Northrop, 1939), they are very similar chemically and physically and may have the same mechanism of activation as is indicated by the urease and phosphatase systems.

A study of the activation energy of an enzyme from several unrelated species must be made to determine whether or not the identity of activation energy for a single enzyme from different species is a general phenomenon. For some enzyme systems it seems likely that the activation energy is independent of the species, since for a number of physiological processes the μ values are the same for many different organisms and probably can be referred to the underlying pacemaker enzyme-catalyzed reactions which determine physiological rates (Gould and Sizer, 1938; Hadidian and Hoagland, 1939).

SUMMARY

The kinetics of urea hydrolysis have been studied with urease partially purified from two different samples of soy bean flour. The course of the reaction was followed by measuring CO_2 evolu-

tion with the Barcroft manometer. At all temperatures CO_2 liberation is a linear function of elapsed time.

Over the temperature range from 0.2–50° the data are in accord with the Arrhenius equation in which the energy of activation is either 8700 or 11,700 calories per gm. mole, depending on the composition of the digest. With certain enzyme preparations the activation energy was 11,700 calories below and 8700 calories above a critical temperature. The identity of activation energies for the soy bean and jack bean urease-urea system has been pointed out.

When a temperature study was made of hydrolysis by unextracted urease still present in the soy or jack bean, the activation energies were identical with those for the extracted and purified enzymes.

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THE INCREASE IN PHOSPHOLIPID AND TOTAL PHOSPHORUS METABOLISM OF THE KIDNEY FOLLOWING THE ADMINISTRATION OF AMMONIUM CHLORIDE, WITH RADIOACTIVE PHOSPHORUS AS AN INDICATOR

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The ability of the kidney, liver, and intestine to synthesize phospholipid (1) and the presence of metabolic phospholipid in these organs (2-4) have been demonstrated. The finding that the rate of incorporation of radioactive phosphorus into the phospholipid of the kidney, unlike the phospholipid of the liver and intestine, is not affected by feeding fat (4) suggested that the kidney phospholipid might be more concerned with phosphorus metabolism than with fat metabolism. If so, an increase in renal phosphorus metabolism should be accompanied by an increase in the rate of kidney phospholipid synthesis; the latter could be detected by the use of radioactive phosphorus.

In this study, an increase in kidney phosphorus metabolism, as shown by an increase in the urinary phosphorus excretion (maximum increase 61 per cent), was induced by NH_4Cl acidosis. A comparison of the rates of incorporation of radioactive phosphorus into the kidney phospholipid of normal and acidotic rats made it possible to decide whether the kidney phospholipid was concerned with phosphorus excretion. The turnover of phosphorus in the whole kidney was studied to determine whether the non-lipid fractions of the kidney also were involved. The evidence presented below indicates that both the phospholipid and the non-lipid phosphorus fractions of the kidney are concerned equally with phosphorus metabolism, since whenever NH_4Cl induced a urinary phosphorus excretion above the control level, there was a nearly equal increase in turnover of the total phosphorus and the phospholipid of the kidney.

Procedure

Young adult rats ranging in weight from 130 to 180 gm. were used. A 12 hour fasting period, which preceded the feeding, served the double purpose of insuring that the rats were in the postabsorptive state and of decreasing the amount of feces excreted during the experimental period, which was desirable since a separation of feces and urine was necessary.

After the fasting period, the animals were given the solutions by means of a calibrated syringe, a long, blunt syringe needle serving as a stomach tube, and immediately placed in individual cages. Within a group, the controls received 1 cc. of a Na_2HPO_4* solution[1] (5) and the experimental animals received 1 cc. of an NH_4Cl solution which contained the same amount of Na_2HPO_4*. The dose of phosphorus, 8 mg., did not vary from group to group, but the radioactivity of the dose varied from 11,000 counts per minute to 13,800 counts per minute on our scale-of-four Geiger-Müller counters (6), except in Group VI which received 77,800 counts per minute. The dose of NH_4Cl was 100, 150, or 200 mg.

The animals were killed 17 hours after feeding; a blood sample was taken and the kidneys were removed for analysis. The 17 hour period was used because it occurs near the peak of Chaikoff's curve for the incorporation of radioactive phosphorus into the phospholipid of the kidney (4). The effect of acidosis on the time at which this peak is reached is still to be investigated.

The urines were filtered after the volumes had been roughly determined. The number of washings and the volume of water employed in the filtration were identical in each case. Aliquots of the filtrates were taken for the determination of titratable acidity and for an ash, the acid solution of which was used for the determination of phosphorus (7) and the degree of radioactivity.

The kidneys were weighed separately immediately after removal from the body. A lipid extract was made from one kidney, and the phospholipid precipitated according to the usual procedure (8); the degree of radioactivity and the amount of phospholipid (8) were determined. The acid solution of the ash of the other kidney was analyzed for total phosphorus and its radioactivity determined. The citrated blood samples were treated in the manner described for the ashed kidney.

[1] The asterisk indicates radioactive phosphorus.

Analytical determinations were made in duplicate with the exception of a few cases in which there was not sufficient material.

The determinations of the degree of radioactivity of the samples were carried out on a Geiger-Müller counter in the manner in general use in this laboratory (6). The degree of radioactivity

TABLE I

Urine of Control and Experimental Rats

The control rats received Na_2HPO_4*. The experimental rats received Na_2HPO_4* and NH_4Cl. The "high experimental" rats excreted in the urine an amount of phosphorus in excess of the control level. The "low experimental" rats eliminated an amount of phosphorus comparable to the control excretion.

		No. of rats	Range	Mean (A.D.)	Increase over control
			cc.	*cc.*	*per cent*
Urine volume	Control	11	0.8– 3.6	**2.0** ± 0.5	
	Experimental	31	3.6– 8.5	**6.2** ± 1.3	**210**
	High experimental	18	4.5– 8.5	6.8 ± 0.9	240
	Low "	13	3.6– 8.2	5.4 ± 1.5	170
0.1 N acid excreted	Control	21	0.0– 4.1	**2.4** ± 0.7	
	Experimental	34	1.0– 6.4	**4.4** ± 0.8	**83**
	High experimental	21	3.7– 6.4	4.9 ± 0.6	104
	Low "	13	1.0– 5.0	3.6 ± 0.9	50
			mg.	*mg.*	
P excreted	Control	21	7.1–14.6	**10.2** ± 1.5	
	Experimental	33	3.8–16.4	**12.5** ± 2.1	**22.6**
	High experimental	21	12.2–16.4	14.0 ± 0.9	37
	Low "	12	3.8–11.2	9.3 ± 1.3	−9.0
			per cent	*per cent*	
P, % of original dose excreted	Control	21	9.9–20.1	**13.1** ± 2.0	
	Experimental	33	4.1–29.2	**17.3** ± 5.4	**32**
	High experimental	21	10.7–29.2	20.7 ± 4.5	58
	Low "	12	4.1–17.9	11.4 ± 2.7	−13

Average values for control and experimental animals are given in bold-faced type.

was expressed as the per cent of the original dose per gm. of kidney, per gm. of blood, and per gm. of renal phospholipid, respectively.

Results

Urinary Phosphorus Excretion—When the data for the individual rats were examined, it appeared that twenty-one of the

thirty-three animals which received NH_4Cl excreted an amount of phosphorus above the control level (37 per cent, Table I); these "high experimental" rats constituted a true experimental group for the study of the relationship between urinary phosphorus excretion and renal phospholipid. The twelve animals in the "low experimental" group excreted on the average slightly less

TABLE II

Blood and Kidneys of Control and Experimental Rats

See note above Table I.

	No. of rats	Range	Mean (A.D.)	Increase over control
Per cent original dose per gm. blood				
				per cent
Control	9	0.07– 0.12	**0.09** ± 0.01	
Experimental	25	0.06– 0.20	**0.14** ± 0.04	**55**
High experimental	13	0.11– 0.20	0.16 ± 0.03	78
Low "	12	0.06– 0.20	0.12 ± 0.04	33
Per cent original dose per gm. renal phospholipid				
Control	17	5.1 – 9.8	**7.1** ± 1.0	
Experimental	35	7.5 –20.6	**11.3** ± 1.8	**59**
High experimental	21	8.9 –20.6	12.2 ± 1.8	72.0
Low "	14	7.5 –12.4	10.0 ± 1.2	29.0
Per cent original dose per gm. kidney				
Control	15	0.33– 0.56	**0.45** ± 0.06	
Experimental	29	0.29– 0.86	**0.64** ± 0.13	**42**
High experimental	16	0.61– 0.86	0.73 ± 0.06	62
Low "	13	0.29– 0.81	0.54 ± 0.15	20

Average values for control and experimental animals are given in bold-faced type.

phosphorus than the control level (−9 per cent). Even though the low experimental group cannot furnish the desired information about the relationship between phosphorus excretion and kidney phospholipid, the average values for all the experimental animals have been presented in bold-faced type in Tables I and II.

The urinary radioactive phosphorus, which parallels the total phosphorus content of the urine, was 58 per cent above the control

level in the case of the high group, and 13 per cent below the control level in the case of the low group.

Diuresis—All the rats which received NH_4Cl showed a marked diuresis, but in this instance also, the percentage increase over the control level for the high experimental group (240) was greater than the corresponding increase for the low group (170).

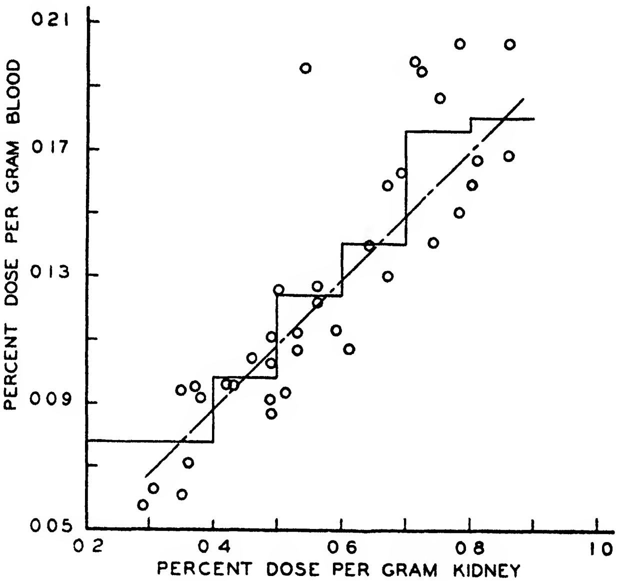

Fig. 1. The relationship between the phosphorus turnover of the blood and of the kidney. The broken line represents the average of all points. The solid line represents the average blood values falling within one interval on the abscissa.

Urinary Acidity—With the exception of two rats in the low group, NH_4Cl administration caused an increase in the titratable acidity of the urine; *e.g.*, the high experimental group excreted 104 per cent more acid than the controls. pH measurements also indicate a greater hydrogen ion excretion for the experimental series; the average pH of the urine of six controls was 6.6 and of eighteen experimental rats 5.9.

P:P Ratio of Blood and Kidneys*—The doses of Na_2HPO_4*, with and without NH_4Cl, affected neither the amount of kidney phos-

pholipid nor the total amount of phosphorus in the blood and kidneys; the analytical values for both the controls and experimental rats were practically identical with those found for normal untreated animals. Table II indicates, however, that the P*:P ratios in the renal phospholipid, in the blood, and in the whole kidney, were markedly increased by the acid-producing salt; the percentage increases for the high experimental group over the control average were 72, 78, and 62 respectively. Figs. 1 and 2,

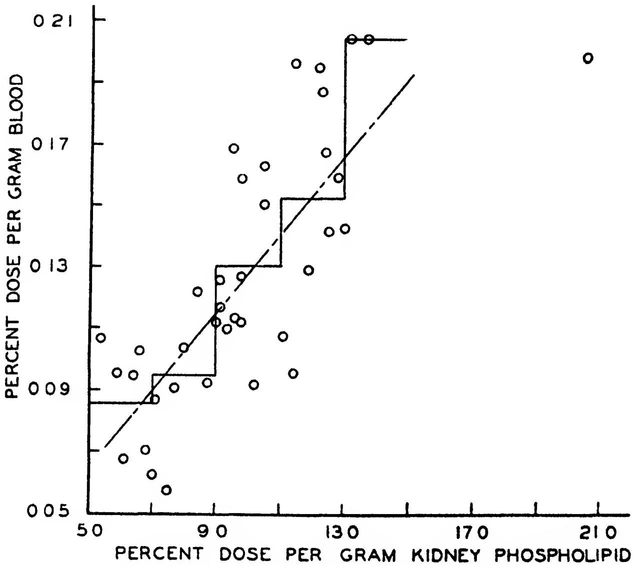

FIG. 2. The relationship between the phosphorus turnover of the blood and of the kidney phospholipid. The broken line represents the average of all points. The solid line represents the average blood values falling within one interval on the abscissa.

in which the radioactivity of the blood has been plotted against the total radioactivity of the kidney and the radioactivity of the renal phospholipid, respectively, show that the P*:P ratio of the blood is proportional to the P*:P ratio of the kidney and also to the P*:P ratio of the kidney phospholipid. Therefore, the increase in the P*:P ratio shows (1) that NH_4Cl caused an increase in phosphorus turnover and (2) that the increase in turnovers of the phospholipid and of the total phosphorus of the kidney were equal.

DISCUSSION

The animal organism which has absorbed NH_4Cl is faced with the necessity of rapidly eliminating an equivalent amount of acid, since the ammonium part of the molecule would be treated as a product of protein metabolism and converted into urea in the liver. The majority of the animals responded to this need by excreting excess phosphorus in the urine, which was accompanied by an increase in the phosphorus metabolism of the blood, kidney, and kidney phospholipid. The phosphorus metabolism and the phospholipid metabolism of the kidney were increased to about the same extent.

The study of the total phosphorus and phospholipid turnovers of the liver in three control and six experimental rats indicates that the liver phosphorus may not be increased by acidosis.

These results indicate that the kidney phospholipid is concerned with the regulation of blood and body acidity by renal excretion of acid phosphate, and also offer an explanation of the rapid turnover of kidney phospholipid during fat absorption which has been noted by Artom *et al.* and Chaikoff *et al.* (3, 4).

SUMMARY

1. A comparison was made of the radioactive phosphorus content of the blood, kidneys, and urine of rats which were fed Na_2HPO_4* with and without NH_4Cl.

2. NH_4Cl acidosis in rats induced an increase in the volume, acidity, total phosphorus, and radioactive phosphorus content of the urine, and an increased phosphorus turnover in the kidney phospholipid, in the whole kidney, and in the blood.

3. These results lend support to the view that the kidney phospholipid is concerned in the regulation of blood and body acidity by the kidney, and explain the increased turnover in kidney phospholipid early in fat absorption.

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Physics for radioactive phosphorus. Radioactive phosphorus was also supplied by the Department of Physics of the University of Michigan and by the Radiation Laboratory of the University of California. This work was aided in part by a grant from the Rockefeller Foundation.

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STUDIES IN PROTEIN METABOLISM

XII. THE CONVERSION OF ORNITHINE INTO ARGININE IN THE MOUSE*

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Ornithine is not a constituent of protein but is obtained as a degradation product from arginine. Krebs and Henseleit (1) have demonstrated that ornithine, when added to liver slices, increases urea production, and have postulated the formation of arginine as an intermediate in the process. The demonstration of isotopic nitrogen in the amidine group of arginine from body proteins after the ingestion of isotopic ammonia (2) or isotopic amino acids (3) has been interpreted as being the result of the same chemical reaction. It was suggested that the arginine present in protein linkage is continuously involved in such reactions, probably in conjunction with successive liberation and reintroduction of arginine molecules.

We have now followed directly the conversion in mice of ornithine into arginine and the introduction of the latter into proteins. Adult mice of constant weight kept on a stock diet containing 15 per cent casein were given deuterium containing *dl*-ornithine for a period of 9 days. The arginine isolated from the proteins of the animals contained 0.88 atom per cent deuterium, corresponding to 1.02 atom per cent in the corresponding ornithine. As the isotopic ornithine administered contained 13.6 atom per cent deuterium, at least 7.5 per cent of the arginine in the total protein of the animal must have been derived from the free ornithine

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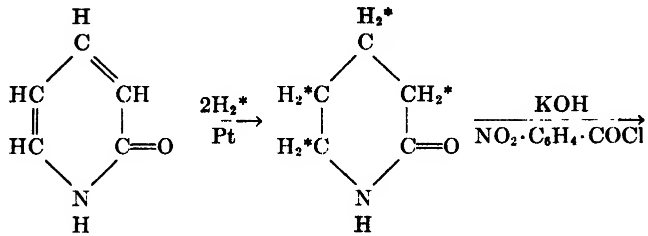
† Rockefeller Foundation Fellow, 1938-39.

administered. *This finding establishes the occurrence in normal adult animals of (a) the conversion of ornithine into arginine, and (b) the replacement of arginine in protein linkage by free arginine.* The latter process may either have occurred in the course of continuous breakdown and resynthesis of proteins, or by merely replacing amino acid molecules. Such reactions have been discussed recently (3) in connection with the feeding of *l*(−)-leucine.[1]

The deuteroornithine was prepared by hydrogenating α-pyridone (I) with deuterium gas to deutero-α-piperidone (II). This was hydrolyzed and δ-aminovaleric acid isolated as its *m*-nitro-



I II

$$\text{III. } NO_2\cdot C_6H_4\cdot CO\cdot NH\cdot CH_2^*\cdot CH_2^*\cdot CH_2^*\cdot CH_2^*\cdot COOH \xrightarrow[Br_2]{P}$$

$$\text{IV. } NO_2\cdot C_6H_4\cdot CO\cdot NH\cdot CH_2^*\cdot CH_2^*\cdot CH_2^*\cdot \underset{Br}{CH^*}\cdot COOH \xrightarrow[\text{hydrolysis}]{NH_3}$$

$$\text{V. } NH_2\cdot CH_2^*\cdot CH_2^*\cdot CH_2^*\cdot \underset{NH_2}{CH^*}\cdot COOH$$

The asterisks designate atoms marked with isotope.

benzoyl derivative (III). It was brominated according to classical procedures to α-bromo-δ-aminovaleric acid (IV) and after treatment with ammonia the *m*-nitrobenzoyl group was removed with boiling hydrochloric acid. The *dl*-ornithine was obtained as the monohydrochloride (V) and contained 12.5 atom per cent deuterium, which was stably bound, as it was not removed by alkaline hydrolysis in Reaction II to III nor by acid hydrolysis in Reaction IV to V.

[1] In an earlier paper from this laboratory it was mentioned in the discussion of arginine metabolism that arginase does not act on this amino acid while it is in protein linkage. According to recent reports (Kraus-Ragins (4)) the enzyme may slowly attack protein-bound arginine.

EXPERIMENTAL

m-Nitrobenzoyldeutero-δ-Aminovaleric Acid — α-Pyridone (2-hydroxypyridine) was prepared from coumalic acid according to von Pechmann and Baltzer (5). It melted at 106–107°. 12 gm. in 12 cc. of dry acetic acid[2] were shaken with deuterium gas in the presence of 1.2 gm. of active platinum at 100° in a hydrogenating vessel surrounded by a steam jacket. When 2 moles of gas had been absorbed (4 hours), ordinary water was added, platinum was filtered off, and a trace of hydrogen sulfide was added (to inactivate any trace of catalyst that might induce exchange of deuterium with ordinary hydrogen in the subsequent operations). Most of the acetic acid was distilled off *in vacuo*, and the residue boiled for 3 hours under a reflux with 120 cc. of 15 per cent sodium hydroxide. The solution was made up to 500 cc., 25 gm. of sodium bicarbonate were added, and 36 gm. of *m*-nitrobenzoyl chloride in 100 cc. of ether were added in four portions over 2 hours with vigorous stirring, which was continued for 2 more hours. The ether layer on evaporation left a crystalline residue consisting of *m*-nitrobenzoylpiperidone. This was heated on the water bath for 30 minutes in 110 cc. of 2 per cent sodium hydroxide solution, when all went into solution. This was combined with the alkaline solution obtained from the benzoylation. It was acidified with hydrochloric acid, and the precipitate was filtered and washed with cold water and ether (which removed *m*-nitrobenzoic acid). It was dissolved in aqueous sodium carbonate and precipitated with acid. The substance melted at 134° and was identical with *m*-nitrobenzyol-δ-aminovaleric acid described by Fischer and Zemplén (6).

The yield was 83.5 per cent. For deuterium analysis a sample was recrystallized from 60 per cent ethanol. It contained 14.6 atom per cent deuterium.

[2] Acetic acid contains 1 atom of exchangeable hydrogen. The hydrogen introduced into pyridone is thus an equilibrium mixture of deuterium and the active hydrogen from acetic acid. The deuterium content in the ornithine is decreased by the amount of acetic acid present during hydrogenation. We have tried, without success, the hydrogenation of pyridone in non-polar solvents. In order to introduce the theoretical amount of deuterium into piperidone it would be necessary to conduct hydrogenation in CH_3COOD. We did not use this solvent, as a higher deuterium content in the ornithine was unnecessary.

dl-Deuteroornithine Monohydrochloride—*m*-Nitrobenzoyl-δ-aminovaleric acid was brominated according to Fischer and Zemplén, and the crude α-bromo derivative, melting at 90–100°, was obtained in 65 per cent yield. A sample was recrystallized from 60 per cent ethanol and melted at 125°. The crude material was used for the preparation of ornithine.

14 gm. of the *m*-nitrobenzoyl-δ-amino-α-bromovaleric acid were dissolved in 100 cc. of 28 per cent ammonia, and the solution was saturated with ammonia gas at 0°. After it had stood for 48 hours, ammonia was again passed through and again left for 48 hours. After removal of ammonia and water the residue was refluxed for 18 hours with 350 cc. of 20 per cent hydrochloric acid. Nitrobenzoic acid was removed by extraction with ether, the aqueous solution evaporated to dryness, redissolved in water, cleared with charcoal, and again evaporated. The residue was extracted with a total of 100 cc. of 95 per cent ethanol, whereby most of the ammonium chloride was removed. The monohydrochloride of ornithine was precipitated by the addition of 3.5 cc. of pyridine. The yield was 5.4 gm. It was further purified by dissolving in water and precipitating with 95 per cent ethanol. The compound contained 16.35 per cent N (calculated, 16.46 per cent). The hydrochloride contained 12.5 atom per cent deuterium. Hence the ornithine contained 13.6 atom per cent deuterium.

The *m*-nitrobenzoyl-δ-aminovaleric acid, from which the ornithine was prepared, contained 14.6 atom per cent deuterium. During the synthesis 1 hydrogen atom was substituted by an amino group which contained normal hydrogen. If no other loss of deuterium had occurred in any of the manipulations, the ornithine should have contained 14.9 atom per cent.

Feeding Experiment—Ten male mice of 24 gm. average weight were kept on a diet consisting of 15 per cent casein, 68 per cent corn-starch, 5 per cent yeast, 4 per cent salt mixture (7), 2 per cent cod liver oil, and 6 per cent Wesson oil. To 260 gm. of total diet were added 2.90 gm. of deuteroornithine monohydrochloride. The animals consumed this mixture within 9 days. They had not changed their initial weight. They were killed, and the intestinal tracts were removed. The remaining bodies were minced, exhaustively extracted with trichloroacetic acid, and hydrolyzed for 48 hours with 20 per cent sulfuric acid. The solution was shaken with ether to remove fatty acids, made alkaline with barium hy-

droxide, filtered, freed of excess barium with sulfuric acid, and allowed to stand for several days. Precipitated tyrosine was removed; the filtrate was acidified and treated with flavianic acid, when 4.03 gm. of flavianate were obtained. This was recrystallized from a large volume of water, decomposed with concentrated hydrochloric acid, the flavianic acid removed, and the mother liquor after treatment with charcoal repeatedly brought to dryness. The residue was dissolved in a small amount of water, and arginine monohydrochloride precipitated with absolute alcohol and pyridine. It was again precipitated from water with alcohol, when it crystallized in long needles. 1.39 gm. were obtained. Found, N (Kjeldahl) 26.4 (calculated, 26.6). The hydrochloride contained 0.82 ± 0.02 atom per cent deuterium, corresponding to 0.88 atom per cent in the free arginine. As all the deuterium must have been in the ornithine moiety, the latter contained 1.02 atom per cent deuterium.

SUMMARY

A method is described for the synthesis from α-pyridone of ornithine containing stably bound deuterium.

Ten mice of constant weight were given for 9 days *dl*-ornithine containing 13.6 atom per cent of stably bound deuterium. The animals were killed and arginine isolated from the total proteins. It contained 0.88 atom per cent deuterium, indicating that at least 7.5 per cent of the total arginine of the animals was derived from ornithine. The finding establishes the occurrence of two reactions: ornithine is continuously converted in mice into arginine; and arginine in protein linkages is continuously replaced by free arginine.

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STUDIES ON THE METABOLISM OF CREATINE AND CREATININE

III. FORMATION OF CREATINE BY ISOLATED RAT TISSUES*

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The precursors, mechanism, and site of creatine formation are still uncertain, even though they have received the attention of investigators for many years. An examination of the results on creatine formation summarized in Hunter's monograph (1928) shows the many contradictions in this field. In the 10 years which have elapsed since the publication of this work, few experiments *in vivo* have been performed which clarify the problem. On the other hand, several workers have reported recently the synthesis of creatine by tissues *in vitro*. The observations of Borsook and Jeffreys (1935) indicate that creatine is formed by liver, kidney, diaphragm, and intestine, and that the rate of formation can be increased in liver by the addition of a complete hydrolysate of egg albumin. Fisher and Wilhelmi (1937) observed an increase in creatine after perfusing the isolated rabbit heart with arginine. Bach (1939) reported an apparent increase in creatine after incubation of chopped rat heart with glycocyamine and glycine.

The specificity of the methods employed in the above investigations is open to some question. We present in this communication results on creatine formation by rat tissues *in vitro*, obtained by employing the *specific, enzymatic* method of Miller, Allinson, and Baker (1939) for the estimation of creatine. The capacity of various tissues to form creatine, without addition of possible precursors, has been observed.

EXPERIMENTAL

The rats used in these experiments were normal, adult males. For purposes of comparison, a few experiments were performed

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with adult females. The animals were killed by decapitation, and the tissues were rapidly excised. In most of the experiments the tissues were cut into small fragments on a cold plate after removal of adherent fat and blood. For experiments with sliced tissue, slices of uniform thickness (0.3 to 0.4 mm.) were prepared by free-hand section, and stored in a moist chamber until ready for use. Excess moisture was removed from the slices with filter papers before weighing. The experiments were performed *with no added substrate* in Ringer-phosphate or Ringer-bicarbonate medium, prepared according to Krebs (1931). Suitable amounts of tissue were rapidly weighed and immersed in 5 cc. of medium, contained in 50 cc. Erlenmeyer flasks. The contents of the flasks were saturated with oxygen or oxygen-carbon dioxide mixture (or nitrogen-carbon dioxide for anaerobic experiments). The flasks were stoppered and shaken in a water bath at 38° for 2 to 4 hours. The pH of the medium remained relatively constant throughout the entire incubation period. Depending on the amount of tissue used, either 10 or 20 cc. of 2 N sulfuric acid were pipetted into the flasks, and the estimation of creatine was carried out as described by Miller, Allinson, and Baker. All experiments were set up in duplicate, and the analysis of each sample was performed in duplicate. Samples of tissue were killed with sulfuric acid at the start of the experiment to give the *initial* creatine concentration. The value obtained *after* incubation represents the *final* creatine concentration. The difference between these two values is the amount of creatine synthesized by the tissue. Since, in this method, the tissue cannot be removed for drying at the end of the experimental period, all results are expressed in terms of original wet weight of tissue.

Results

Typical results obtained with various rat tissues in the absence of added substrate are given in Tables I and II. All values represent the average of determinations on duplicate samples of tissue. The data show that only liver and kidney produce significant amounts of creatine from precursors *normally present* in the tissue. The percentage increases in kidney and liver are very striking, averaging approximately 10 to 20 per cent for the former and 10 to 35 per cent for the latter tissue. No significant *percentage* in-

creases were observed in brain, testis, spleen, and muscle (cardiac, skeletal, and intestinal muscles were studied). As can be seen from Table I, very slight absolute increases were occasionally observed in muscle creatine. However, the high initial creatine content of this tissue interferes with the precise estimation of small increases of creatine. Nevertheless, even though it is question-

TABLE I

Formation of Creatine by Rat Muscle, Brain, Testis, and Spleen

Tissue*	Buffer	Incubation period	Creatine, mg. per 100 gm. wet weight of tissue		Increase
			Initial	Final	
		hrs.			*per cent*
Heart muscle	Phosphate	2	202	207	+2
	"	2	221	217	−2
	"	2	198	198	0
	Bicarbonate	4	174	174	0
Diaphragm	"	3	407	410	+1
	"	6	409	415	+2
	"	4	322	327	+2
Intestinal muscle	"	4	96	95	−1
	"	4	101	100	−1
Brain	"	3	119	122	+3
	"	6	119	121	+2
	"	4	149	147	−2
Testis	Phosphate	2	301	302	<1
	Bicarbonate	2	302	294	−3
	"	4	328	328	0
Spleen	"	3	19.8	19.9	<1
	"	6	19.8	20.2	+2
	"	4	18.1	18.0	<1

* All experiments were performed with chopped tissue.

able that the few positive results obtained on muscle tissue represent true production of creatine, our data cannot preclude it.

As shown in Table II, the amount of creatine synthesized per 100 gm. of wet weight of kidney tissue is the same for male and female rats. It is further shown that chopped and sliced kidney tissues are equally efficient in the production of creatine.

The following experiment suggests that an enzyme system is involved in the formation *in vitro* of creatine. Kidney tissue,

inactivated by heating at 80° for 10 minutes, produced no creatine upon subsequent incubation at 38° for 4 hours; unheated kidney tissue from the same animal produced 4.4 mg. per 100 gm. Furthermore, the synthesis is completely inhibited by 10^{-3} M potassium cyanide,[1] and partially inhibited by anaerobiosis.

TABLE II

Formation of Creatine by Rat Liver and Kidney

Tissue	Buffer	Incubation period	Creatine, mg. per 100 gm. wet weight of tissue		Increase	Remarks
			Initial	Final		
		hrs.			*per cent*	
Liver	Phosphate	2	4.6	6.1	+33	
	Bicarbonate	3	7.1	8.0	+13	
	"	4	5.5	6.6	+20	
	"	4	9.9	13.3	+34	
	"	2	34.6	38.4	+11	Male
	"	3	52.0	55.6	+7	"
	"	6	52.0	58.9	+13	
	"	4	29.3	34.6	+18	"
	"	4	38.7	46.4	+15	Female
	"	3	26.3	30.3	+15	
	"	4	24.0	28.5	+19	
Kidney	Phosphate	4½	44.3	48.1	+9	Female
	Bicarbonate		45.2	54.3	+16	
	"	4	36.1	42.8	+19	Chopped. Male
	"		34.3	40.8	+19	Sliced. "
	"	4	31.3	36.8	+18	Aerobic. Female
	"		31.3	33.9	+8	Anaerobic. "
	"	4	55.1	59.4	+8	Control. Male
	"			53.5		10^{-3} M KCN. "

That the increase is due almost entirely to creatine, and not to creatinine, was shown by an experiment on kidney tissue in which

[1] Cyanide interferes with the Jaffe reaction. Therefore, it was completely removed by distillation before the determination of creatine.

both creatine and creatinine were determined after a 4 hour incubation period. (Creatinine was determined by the method of Miller, Allinson, and Baker.) The increase in creatine *plus* creatinine was 7.7 mg. per 100 gm. Of this, 7.5 mg. were creatine, and only 0.2 mg. creatinine.

It is interesting to note that the *absolute* quantities of creatine produced are approximately 1 to 2 mg. per 100 gm. of kidney tissue per hour, and 0.3 to 0.8 mg. per 100 gm. for liver. Since it is likely that the process is considerably more efficient *in vivo* than in tissue slices and minces, it is possible that the kidney and liver play an important rôle in the production of creatine under normal physiological conditions.

SUMMARY

The formation of creatine by *isolated rat tissues* without added substrate has been studied. Kidney produced 4 to 8 mg. per 100 gm., and liver, 1.2 to 3.2 mg. per 100 gm. in a 4 hour incubation period. These values represent approximately 10 to 30 per cent increases. The process of creatine formation is apparently enzymatic in nature: it is heat-labile and is inhibited by cyanide and by anaerobiosis.

In other tissues studied, cardiac, skeletal, and intestinal muscle, brain, testis, and spleen, no significant *percentage* increases of creatine were found. (It is impossible with present methods to detect small, absolute increases in tissues of high creatine content such as cardiac and skeletal muscle.)

All analyses were performed with a specific, enzymatic method for the determination of creatine.

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THE BIOCHEMICAL BEHAVIOR OF LEAD

I. INFLUENCE OF CALCIUM, PHOSPHORUS, AND VITAMIN D ON LEAD IN BLOOD AND BONE*

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In preliminary papers from this laboratory (1, 2) a marked effect of vitamin D on the lead concentrations of blood and bone of young rats fed lead-containing diets was demonstrated. Groups fed vitamin D contained a higher percentage of lead in the bone ash on low calcium-low phosphorus, high calcium-low phosphorus, and low calcium-high phosphorus types of diets. Vitamin D raised the lead concentrations of the blood of the rats on all the diets except in the group on the high phosphorus-low calcium diet. In the group on the high phosphorus-low calcium diet, the lead concentration of the blood was so much lower than in the other two groups that it could not be determined accurately and any effect of vitamin D could not be demonstrated.

The influence of calcium and phosphorus on the behavior of lead, which was emphasized by several investigators, was also shown in these preliminary reports. In rats fed high calcium-low phosphorus diets, the lead in the blood was markedly higher than in those fed high phosphorus-low calcium diets, despite the fact that the latter diet contained 3 times as much lead as was present in the high calcium-low phosphorus diet.

In the present investigation these studies of the effect of calcium, phosphorus, and vitamin D on the biochemical behavior of lead are extended. The experiments were designed so that quantita-

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tive data of the effect of calcium, phosphorus, and vitamin D on lead deposition, as compared to calcium deposition, could be obtained. The purpose was to clarify the type of relationship that exists between deposition of calcium and lead.

EXPERIMENTAL

Three diets were used in these experiments. The basal diet contained traces of calcium (0.03 per cent) and small amounts of phosphorus (0.262 per cent), to which 1 per cent of basic lead carbonate was added. This diet was used without other additions as a low calcium-low phosphorus diet (Diet A). To this basal

TABLE I

Experimental Lead Diets

Diet	Constituents		Ca	P	Pb
		parts	*per cent*	*per cent*	*per cent*
Low calcium-low phosphorus (basal), Diet A	Yellow corn-meal	70	0.025	0.262	0.82
	Wheat gluten	16			
	Brewers' yeast	10			
	NaCl	1			
	$Pb(OH)_2 \cdot 2PbCO_3$	1			
High calcium-low phosphorus, Diet B	Basal diet	98	1.02	0.262	0.80
	$CaCO_3$	2.5			
High phosphorus-low calcium, Diet C	Basal diet	97.25	0.025	0.857	0.80
	Na_2HPO_4	2.75			

diet 2.5 per cent of calcium carbonate was added to produce a high calcium-low phosphorus diet (Diet B), and 2.75 per cent of anhydrous Na_2HPO_4 was added to produce the high phosphorus-low calcium diet (Diet C). The diets are shown in Table I. The calcium, phosphorus, and lead values of the diet were determined by duplicate analyses. These diets varied with respect to calcium and phosphorus but were similar in lead content. Furthermore, each diet contained the same amount of either calcium or phosphorus as one of the others.

Each diet of the above three was fed to about thirty-four to forty rats. After 5 days on the experimental diets, one-half of each group was given 100 I.U. of vitamin D daily until the end of

the experimental period. The vitamin D was fed by mouth from a pipette in the form of a solution of viosterol in maize oil (0.1 cc. = 100 I.U. of vitamin D).

Albino rats raised in our laboratory from an original Wistar strain were used in all these experiments. The mothers were kept on the stock diet of Bills *et al.* (3). The young rats were usually weaned at 21 days of age, at which time they were placed on the stock diet. The animals chosen for the experiment were from litters of eight to thirteen. Large litters were chosen because they could easily be divided among the various experimental groups. The average initial weight of these rats was about 35 gm. (see Table II), which is below the average weight of animals of this age

TABLE II

Comparison of Mean Initial Weights

Diet	No. of animals	Mean initial weight	Standard deviation of mean	Standard error of mean
		gm.		
A	25	34.9	±6.09	±1.22
" + vitamin D	14	35.3	±4.19	±1.12
B	15	37.7	±5.80	±1 50
" + vitamin D	16	35.1	±6.45	±1.61
C	22	33.5	±3.91	±0.84
" + vitamin D	20	32.0	±6.30	±1.41

from smaller litters. At this average weight (35 gm.) most of the animals were 24 to 26 days of age, with the exception of one litter the age of which was 29 days. The mean initial weight with the standard deviation and the standard error of the mean for each group is presented in Table II. It is seen that the mean weights of the various groups are within the narrow range of 32.0 to 37.7 gm. Thus, it is evident that the various groups were as similar in weight as could be expected in experiments with rats of various litters.

The similarity of heredity, the splitting of litters into various groups, and the identity of initial weights establish a reasonable basis for the comparison of the results in the various groups.

At the end of 29 days the animals were x-rayed and then sacrificed. Blood was obtained by heart punctures or by cutting

of the carotid arteries. To prevent lead contamination the fur of the animals was carefully cut before the blood was drawn. In most cases lead determinations were carried out on the pooled whole blood of two or three animals. In Group C, which received the high phosphorus-low calcium diet (Diet C), the blood of five or six animals was used for a single analysis because of the low lead value in the blood of this group (2). In Groups A and B (fed Diets A and B respectively) in addition to determination of the lead of whole blood, serum calcium, serum inorganic phosphorus, and serum lead were determined. For this purpose the blood of six animals was pooled to provide sufficient serum. Only one serum analysis was made on each group, since the primary objective of these experiments was to obtain the lead value of whole blood. In Group C, more blood was needed for whole blood lead analyses, owing to the low value of the blood lead, and no blood was left for serum analyses. The serum values reported here are more in the nature of preliminary experiments and are not conclusive. The lead values for whole blood reported in this paper are the mean results of six to ten analyses and are therefore significant.

The blood lead was determined by the method of Wilkins *et al.* (4), the serum calcium by the method of Sobel and Sklersky (5), and the serum inorganic phosphorus by the method of Fiske and Subbarow (6).

The femur of each animal was carefully dissected out, freed of all adhering tissue, extracted with alcohol-ether, and then dried at 105° for 24 hours. The bone ash was determined on the dried, fat-free bones by heating to constant weight at 560–600° in an electric muffle furnace. The lead content of the ashed bone was determined by a modification of the method of Willoughby *et al.* (7).

Calculation of Results

(1) $$\%\text{ bone ash} = \frac{\text{gm. weight of ash} \times 100}{\text{gm. weight of fat-free, dried femur}}$$

(2) $$\text{Mg. }\%\text{ lead in dried bone} = \frac{\text{mg. lead in femur} \times 100}{\text{gm. weight of fat-free, dried femur}}$$

(3) $$\text{Mg. }\%\text{ lead in ash} = \frac{\text{mg. lead in femur} \times 100}{\text{gm. weight of ashed femur}}$$

$$(4) \qquad \text{Mg. \% lead in organic bone} = \frac{\text{mg. lead in femur} \times 100}{\text{gm. organic matter in femur}}$$

$$\text{Mg. organic matter in femur} = \text{(total weight)} - \text{(ashed weight)}$$

$$\text{Total weight of femur} = \text{inorganic} + \text{organic matter}$$

$$= \text{weight of ash} + \text{weight of organic matter}$$

The mean results of the bone analyses were evaluated by the statistical methods of Fisher as applied to small samples (8). *P* represents the frequency with which the difference between two means may be due to chance alone.

Influence of Vitamin D

Low Calcium-Low Phosphorus Diet—The results showing the influence of vitamin D on the rats receiving the low Ca-low P diet are presented in Table III. A definite influence of the antirachitic vitamin is shown in several respects. The degree of calcification, as shown by the mean bone ash values, is significantly higher in the group fed vitamin D. The mean ash value was 32.5 per cent in this group and only 27.6 per cent in the group without vitamin D. This difference of mean values gave a *P* of less than 1 part in 100,000, which is a high level of statistical significance. The percentage of lead in the dried bone is more than 3 times as large in the vitamin D group. The value for the group without vitamin D was 164 mg. per 100 gm. of dried bone and for the vitamin D group it was 535. This difference in the amount of lead deposited becomes more marked when the results are expressed as lead per unit of organic matter in the bone, which is a better criterion of the degree of lead deposition. The vitamin D group averaged 800 mg. of lead per 100 gm. of organic matter in the bone, whereas the group without the vitamin D averaged 225 mg. of lead per 100 gm. The relative rates of lead deposition and calcification are distinctly different in the two groups, as shown by the mg. per cent of lead in the bone ash. The amount of lead per unit of ash is almost 3 times as high in the vitamin D group as in the control group. The mg. per cent of lead per 100 gm. of ash in the vitamin D group was 1643, whereas in the group without vitamin D it was 608. This indicates that not only is the degree of deposition of lead higher under the influence of vitamin D but also the relative rates of deposition are higher when compared to the amount of calcification.

The mean value of the lead of whole blood for the non-vitamin D group is 0.29 mg. per 100 cc., whereas the vitamin D group has a mean of 0.77 mg. per 100 cc. A statistical treatment of the values obtained shows that this difference is significant, P being less than 1 in 1000. The serum Ca × P product is higher in the vitamin D group. There was an insufficient number of analyses of serum lead to draw any significant conclusions concerning the influence of vitamin D on serum lead in this group. The serum

TABLE III

Influence of Vitamin D; Low Calcium-Low Phosphorus Diet
(Mean Values)

P = probability that the difference in the mean values between the two groups is due to chance; per cent ash = measure of the degree of calcification; mg. per cent Pb in ash = measure of the relative amounts of lead deposition in bone and calcification; mg. per cent Pb in organic matter = measure of the degree of lead deposition.

	Change in weight	Serum			Whole blood Pb	Analysis of dried, fat-free femora			
		Ca	P	Pb		Ash	Pb	Pb in ash	Pb in organic matter
	gm.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
No vitamin D, 21 rats	+19.3	6.8	6.5	0.10	0.29	27.6	164	608	225
100 I.U. vitamin D daily, 13 rats ...	+26.7	7.0	8.0	0.10	0.77	32.5	535	1643	800
P..........					$<10^{-3}$	$<10^{-5}$	$<10^{-8}$	$<10^{-9}$	$<10^{-9}$

calcium is low in both groups, whereas the phosphorus is nearer the normal and is higher in the vitamin D group. The serum Ca × P product as well as the serum Pb × P product appears to be somewhat higher in the vitamin D group.

High Calcium-Low Phosphorus Diet—The results showing the influence of vitamin D on the rats receiving the high calcium-low phosphorus diet are shown in Table IV. The degree of calcification in the vitamin D group, as indicated by the bone ash, is about twice as great. The mean bone ash value of the vitamin D group was 37.9 per cent, whereas in the group without vitamin D it was

19.3 per cent. The degree of lead deposition is distinctly higher in the vitamin D group, as shown by the mg. per cent of lead in the organic part of the bone, which is 518 mg. per cent in the vitamin D group and 125 mg. per cent in the control group. The per cent increase in deposition of lead is greater than that of calcium, since the amount of lead per unit of ash in the bone is almost 1.5 times as great in the vitamin D group as in the control group. The mg. of lead per 100 gm. of ash in the vitamin D group is 874, whereas in the control group it is 523. All of the last named differences are statistically significant, as shown by the P values in Table IV.

TABLE IV

Influence of Vitamin D; High Calcium-Low Phosphorus Diet (Mean Values)

	Change in weight	Serum			Whole blood Pb	Analysis of dried, fat-free femora			
		Ca	P	Pb		Ash	Pb	Pb in ash	Pb in organic matter
	gm.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
No vitamin D, 13 rats	+22.6	8.8	3.5	0 08	0.51	19.3	101	523	125
100 I.U. vitamin D daily, 16 rats	+27.0	11.4	4 0	0.20	0.77	37.9	317	874	518
P .					0.43	$<10^{-6}$	$<10^{-9}$	$<10^{-4}$	$<10^{-9}$

P = probability that the difference between the two means is due to chance.

There is a slightly higher value for the lead of whole blood in the vitamin D group, 0.77 mg. per 100 cc. for the vitamin D and 0.51 mg. per 100 cc. for the group without vitamin D, but this difference is without statistical significance. As might be expected on a high calcium-low phosphorus diet, the serum calcium tends towards the normal 8.8 mg. per 100 cc. in the group without vitamin D, and 11.4 mg. per 100 cc. in the group fed vitamin D. The serum phosphorus values are distinctly low, even in the vitamin D group (4.0 mg. per 100 cc.). The serum lead appears to be distinctly higher in the vitamin D group (0.20 mg. per 100 cc.), as contrasted to the group without vitamin D, which had a serum

lead value of 0.08 mg. of lead per 100 cc. of serum. These two serum lead values are the results of single analyses on pooled serum of the corresponding groups. Conclusive significance cannot be attached to these single values. Both the Pb × P and the Ca × P products are higher in the vitamin D group.

High Phosphorus-Low Calcium Diet—The results showing the influence of vitamin D on the rats receiving the high phosphorus-low calcium diet are presented in Table V. Here again a definite

TABLE V

Influence of Vitamin D; High Phosphorus-Low Calcium Diet (Mean Values)

	Change in weight	Whole blood Pb		Analysis of dried, fat-free femora: Ash	Pb	Pb in ash	Pb in organic matter
	gm.	*mg. per cent*		*per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
No vitamin D, 21 rats	+15.5	0.28 0.09 0.00 0.07	0.11	27.4	87	335	121
100 I.U. vitamin D daily, 20 rats	+24.0	0.17 0.13 0.00 0.14 0.34 0.35	0.19	34.0	244	729	361
P				$<10^{-9}$	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$

P = probability that the difference between the two means is due to chance.

influence of the antirachitic vitamin is evident. The degree of calcification is definitely higher in the vitamin D group, where the mean ash percentage is 34.0, whereas in the control group it is 27.4. The *P* value of the difference in these means is less than 1 part in 1,000,000,000, which is an extremely high level of significance statistically. This increase of ash under the influence of vitamin D is less than in Group B but somewhat greater than in Group A. The degree of lead deposition is 3 times higher in the vitamin D group than in the control group. The mg. per cent

of lead in the organic matter in the bone is 361 in the vitamin-fed group and only 121 mg. per cent in the control group. The ratio of the deposition of lead over that of calcium is distinctly higher in the vitamin D group, as shown by the amount of lead per unit weight of ash. This is 729 mg. per cent in the vitamin D group and 335 in the group without the vitamin D.

The mean blood lead values are higher in the vitamin D group than in the control group. In some cases the blood lead was so low that it could not be determined accurately on the small

TABLE VI

Influence of Dietary Ca and P in Absence of Vitamin D (Mean Values)

	Change in weight	Serum			Whole blood Pb	Analysis of dried, fat-free femora			
		Ca	P	Pb		Ash	Pb	Pb in ash	Pb in organic matter
	gm.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Low Ca-low P diet, 21 rats . .	+19.3	6.8	6.5	0.10	0.29	27.6	163	607	225
High Ca-low P diet, 13 rats	+22.6	8.8	3.5	0.08	0.51	19.34	101	522	125
Low Ca-high P diet, 21 rats	+15.5				0.11	27.4	87	335	121
P, Diet A *versus* B						$<10^{-9}$	10^{-3}	0.33	10^{-4}
" " " " C						0 87	10^{-5}	10^{-4}	10^{-5}
" " B " "						$<10^{-9}$	0.22	10^{-3}	0.80

P = probability that the difference between the two means is due to chance.

quantities of blood available. Where 0.00 mg. per cent is indicated, it actually represents some value less than 0.1 mg. per cent. In view of this fact it cannot be safely stated that the difference between the vitamin D and the control groups is significant.

Thus it can be seen that vitamin D increases the degree of deposition of lead in all groups from 3- to 4-fold, as measured by the amount of lead per unit of organic material in the bone. It also increases the degree of calcification in all groups, as measured by the percentage bone ash. This increase in bone ash is greater

in the high calcium-low phosphorus group than in either of the other two groups. Vitamin D increases the deposition of lead in these diets to a greater degree than that of calcium, as shown by the mg. per cent of Pb per unit of ash. This increase of the relative rates was least in the high calcium-low phosphorus group, where the increase in calcification is the greatest.

Vitamin D distinctly increases the lead of whole blood in the low calcium-low phosphorus group. In the high calcium-low phosphorus group, vitamin D has no significant influence on the blood lead, a finding which is not wholly unexpected on a low phosphorus-high calcium-high Pb diet. On the low calcium-high phosphorus diet, the vitamin D group shows a higher average of lead in whole blood than the control. However, owing to the low values of lead in the blood in this group, it is difficult to attach significance to this difference.

There was an insufficient number of serum analyses to draw definite conclusions. However, both the Ca × P product and the Pb × P product appear to be higher under the influence of vitamin D in Groups A and B. This may be an explanation for the increase in the deposition of calcium and lead. Further investigations will be necessary to evaluate this relationship between serum Pb × P product and lead deposition.

Influence of Dietary Calcium and Phosphorus

Table VI shows the influence of calcium and phosphorus in the absence of vitamin D. The degree of calcification is significantly lower in the high calcium-low phosphorus group than in either of the two other groups, which have about the same degree of calcification. The degree of deposition of lead is higher in the low calcium-low phosphorus group than in either of the other two groups. Groups B and C have about the same degree of lead deposition. Thus, the addition of either calcium or phosphorus to the low calcium-low phosphorus diet causes a decrease in lead deposition. The ratio of the deposition of lead over calcium, as shown by the mg. per cent of lead in ash, does not change significantly when calcium is added to Diet A but is significantly lower when phosphorus is added to Diet A.

The blood lead becomes progressively higher as the Ca:P ratio goes up, being highest in the group fed the high calcium-low

phosphorus diet and lowest in the group fed the high phosphorus-low calcium diet. Thus, addition of calcium increases the value of the lead in the whole blood, whereas addition of phosphorus decreases the blood lead. The serum calcium and phosphorus reflect the dietary calcium and phosphorus. In Group B the serum calcium is almost normal, whereas the serum phosphorus is distinctly below normal. In Group A, the serum calcium is distinctly below normal, whereas the phosphorus is almost normal.

TABLE VII

Influence of Dietary Ca and P in Presence of Vitamin D (Mean Values)

	Change in weight	Serum			Whole blood Pb	Analysis of dried, fat-free femora			
		Ca	P	Pb		Ash	Pb	Pb in ash	Pb in organic matter
	gm.	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Low Ca-low P diet, 13 rats	+26.7	7.0	8.0	0 10	0.77	32.5	535	1643	800
High Ca-low P diet, 16 rats	+27.0	11.4	4.0	0.20	0 77	37.9	317	874	518
Low Ca-high P diet, 20 rats	+24.0				0.19	34.0	244	729	361
P, Diet A *versus* B						0.02	$<10^{-8}$	$<10^{-9}$	$<10^{-3}$
P, Diet A *versus* C						0.19	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$
P, Diet B *versus* C						0.02	$<10^{-3}$	0.11	$<10^{-9}$

P = probability that the difference between the two means is due to chance.

The serum lead is about the same in each group. Both the serum Ca × P and the serum Pb × P products are higher in the low phosphorus-low calcium group, and so is the deposition of calcium and lead higher in this group than in the high calcium-low phosphorus group. These data on serum are suggestive but not conclusive. Additional data are necessary.

The influence of dietary calcium and phosphorus in the presence of vitamin D is shown in Table VII. The degree of calcification is highest in the high calcium-low phosphorus group. This dif-

ference of means between Group B and the other two groups is statistically significant, if the usual statistical convention of significance is used, where a difference between two means is significant when P is 0.05 or less. In our opinion the difference of means in the degree of calcification in these groups is not conclusive. The degree of deposition of lead here, again, is highest in the low calcium-low phosphorus group. Addition of calcium to Diet A depressed the degree of lead deposition, and addition of phosphorus to Diet A depressed it even more.

The ratio of the deposition of lead over calcium, as shown by the lead per unit of ash, is distinctly higher in the low calcium-low phosphorus group than in either of the other two groups. Addition of either calcium or phosphorus in the amounts used in this experiment depressed the relative rates of deposition of lead and calcium to about the same degree.

The lead in whole blood is much lower in the high phosphorus-low calcium group than in either of the two other groups. The addition of calcium to Diet A does not influence the lead in whole blood to any great extent, but addition of phosphorus to Diet A depresses the blood lead. The serum calcium and phosphorus reflect the dietary ratios of Ca:P. In the group on the low calcium-low phosphorus diet serum calcium is low and serum phosphorus is normal, whereas in the group on the high calcium-low phosphorus diet serum calcium is normal and the phosphorus is definitely low in spite of the presence of vitamin D. The serum lead of the rats on the high calcium-low phosphorus diet is higher than that of the rats on the low calcium-low phosphorus diet. Interestingly enough, the Pb $\times$ P product was the same in both types of diets. However, the lead deposition was higher in the low calcium-low phosphorus group than in the high calcium-low phosphorus group. Although the serum Ca $\times$ P product was higher in Group A than in Group B, the degree of calcification of Group B was higher than in Group A. However, this difference in percentage ash in Groups A and B is not considered conclusive by the authors.

Thus, it can be seen from Tables VI and VII that the calcium and phosphorus content of the diet plays a definite rôle in the deposition of lead as well as of calcium. The degree of lead deposition is highest in the low calcium-low phosphorus group

among the three groups fed vitamin D as well as among the three groups not receiving vitamin D. However, the lowest degree of lead deposition in the group fed vitamin D (Group C) is still higher than the highest (Group A) among the groups not receiving vitamin D. Addition of either calcium (Group B) or phosphate (Group C) to the low Ca-low P diet reduces the degree of lead deposition. This reduction is greater in Group C than in Group B among the vitamin-fed animals, but is approximately of the same degree among the animals without vitamin D.

The degree of calcification is highest in the high calcium-low phosphorus group in the group fed vitamin D and is the lowest in the same group, in the absence of vitamin D. The degree of calcification is in all cases below normal. (The mean ash of normal animals of this age is about 56 per cent.) The degree of calcification is even less than that of the rats at the beginning of the experiment. (The mean ash of 23 day-old rats from our colony is 41.9 per cent.)

The addition of phosphorus to the low calcium-low phosphorus diet has a marked depressing effect on whole blood lead, both in the presence and absence of vitamin D. The addition of calcium to Diet A causes an increase in the lead in whole blood in the absence of vitamin D. However, in the presence of vitamin D, the lead in whole blood remains almost stationary when compared to that of the group with vitamin D, fed the low calcium-low phosphorus diet. The serum calcium and inorganic phosphorus appear to reflect the dietary calcium and phosphorus ratios, which are not corrected to any marked degree by feeding vitamin D. This may be attributed to the complicating presence of lead in the diet. In the absence of lead, vitamin D would restore the normal serum Ca $\times$ P product by raising the calcium of the low calcium-low phosphorus group and by raising the phosphorus of the high calcium-low phosphorus group (9, 10).

Deposition of Lead and Calcium As Indicated by Total Amount of Lead and Ash in Femurs

In Tables III to VII the degree of calcification was presented as measured by the *ratio* of ash to total bone weight. The degree of lead deposition was measured as the *ratio* of lead to total or organic bone weight. In Table VIII the absolute amounts of

deposition of calcium and lead are presented. The data presented show the total amount of calcification and the total amount of lead deposited in the femurs at the end of the experimental period.

It can be seen from Table VIII that the femurs of the group fed vitamin D on each diet contain distinctly greater amounts of ash than the femurs of the groups not receiving the antirachitic vitamin. This increase due to vitamin D was 3.70 mg. in Group A, 20.12 mg. in Group B, and 5.04 mg. in Group C. In other words the addition of calcium to Diet A greatly increased the effect of vitamin D on the amount of calcification, but the addition of phosphorus to Diet A increased the effect of vitamin D to a small degree only. In Diet A the effect of vitamin D in increasing calcification is the least among the three groups.

In addition to increasing the amount of bone ash in these three groups, vitamin D also causes an increase in the amount of lead deposited in the femurs in each group. This increase due to vitamin D was 251.2 γ of Pb in Group A, 197.9 γ of Pb in Group B, and 94.5 γ of Pb in Group C. Thus, the addition of calcium to Diet A decreases the effect of vitamin D on the amount of lead deposited; the addition of phosphorus to Diet A decreases the effect of vitamin D even more.

The ratio of the increase of Pb over the increase of ash in each group under the influence of vitamin D is as follows: ΔPb/Δ ash for Group A, 251.2/3.7 = 67.9; Group B, 197.9/20.12 = 9.84; Group C, 94.5/5.04 = 18.75. ΔPb = mean Pb (in γ) per femurs of the group fed vitamin D minus mean Pb(in γ) per femurs of the same group without vitamin D; Δ ash = mean ash (in mg.) per femurs of the group fed vitamin D minus the mean ash (in mg.) per femurs of the group without vitamin D.

It can be seen that ΔPb/Δ ash is about 7 times as great in Group A as in Group B and is about 3.5 times as great in Group A as in Group C. Thus, addition of calcium or phosphorus to Diet A decreases the influence of vitamin D on the ratio of the increase of deposition of lead over that of calcium.

To sum up, the addition of calcium to Diet A increases the effect of vitamin D on calcification and decreases the effect of vitamin D on lead deposition. The addition of phosphorus to Diet A increases the influence of vitamin D on calcification slightly, but decreases the effect on the deposition of lead of vitamin D

to a marked extent. These changes are quantitatively expressed in the $\Delta Pb:\Delta$ ash ratios.

TABLE VIII

Influence of Calcium, Phosphorus, and Vitamin D on Absolute Weight of Lead and Ash in Femora (Mean Values)

The lead and ash values are those found per whole femur.

	Pb	Ash	P values							
			Pb	Ash	Pb	Ash	Pb	Ash	Pb	Ash
			Diet A + vitamin D		Diet B		Diet B + vitamin D		Diet C	
	γ	*mg.*								
Diet A, 21 rats	103.0	17.76	$<10^{-9}$	10^{-3}	0.01	10^{-4}			10^{-5}	0.08
Diet A + vitamin D, 13 rats	354.2	21.46					0.01	10^{-3}		
Diet B, 13 rats	68.4	13.06					$<10^{-9}$	10^{-8}	0.90	0.01
Diet B + vitamin D, 16 rats	266.3	33.18	0.01	10^{-3}	$<10^{-9}$	$<10^{-8}$				
Diet C, 21 rats	51 8	15.88				0.01				
Diet C + vitamin D, 20 rats	146.3	20.92	$<10^{-9}$	0.75			$<10^{-5}$	10^{-4}	10^{-7}	10^{-5}

P = probability that the difference between the two means is due to chance.

Diet A = low calcium (0 03 per cent), low phosphorus (0.262 per cent), lead (0.82 per cent); Diet B = Diet A + 2.5 per cent $CaCO_3$ = high calcium (1.02 per cent), low phosphorus (0.26 per cent), lead (0.80 per cent); Diet C = Diet A + 2.75 per cent Na_2HPO_4 = low calcium (0.03 per cent), high phosphorus (0.857 per cent), lead (0.80 per cent). Vitamin D groups received 100 I.U. of vitamin D daily in addition to the diet.

The mean weight of the bone ash of a group of rats, 23 days of age, was 23.4 mg. These rats corresponded in size and weight to the experimental animals *before* they were placed on the experimental diets.

Influence of Calcium Added to Diet A—In the absence of vitamin D, the addition of calcium to Diet A caused a decrease of 4.70

mg. of bone ash and 34.6 γ of Pb in the femur. In the presence of vitamin D, the addition of calcium caused an increase of 11.72 mg. of bone ash and a decrease of 87.9 γ of Pb. The ratio, ΔPb:Δ ash, of the change in lead over that of bone ash is given in Table IX.

Calcium added to Diet A decreases the deposition of lead both in the presence and absence of vitamin D. Calcification in the absence of vitamin D is also decreased, whereas in the presence of vitamin D it is increased; so that in the vitamin D group, deposition of calcium and lead go in opposite directions under the influence of calcium added to the low calcium-low phosphorus diet. This is clearly shown in the ΔPb:Δ ash ratios, which are

TABLE IX

Ratio of Change in Lead over That of Bone Ash under Influence of Dietary Calcium

	No vitamin D		Vitamin D group	
	Ash	Pb	Ash	Pb
	mg.	γ	*mg.*	γ
Diet A	17.76	103.0	21.46	354.2
" " + 2.5% $CaCO_3$	13.06	68.4	33.18	266.3
Δ(mean values of Group B − A)	−4.70	−34.6	+11.72	−87.9
$\frac{\Delta Pb}{\Delta\ ash}$	+7.36		−7.48	

+7.36 in the absence of vitamin D and −7.48 in the presence of vitamin D. *In this case, a dietary factor which promotes calcification (the additional $CaCO_3$) causes increased calcification and a definite decrease in deposition of lead.*

Influence of Phosphorus Added to Diet A—In the absence of vitamin D, the addition of phosphorus to Diet A causes a negligible decrease of 1.88 mg. of bone ash and a significant decrease of 51.2 γ of lead in the femur. In the presence of vitamin D, the addition of phosphorus to Diet A causes a negligible decrease of 0.54 mg. of bone ash and a significant decrease of 208.9 γ of lead in the femur. (For the statistical significance of these changes, refer to the *P* values of Table VIII.) Thus, the addition of phosphorus to Diet A has practically no effect on calcification

but markedly decreases the amount of lead deposited in the femurs.

Comparison of Path of Deposition of Calcium and Lead in Bones—It is seen in Table VIII that the amount of ash in the femur of a group of rats, 23 days of age, is higher than the bone ash of the experimental animals at the end of the experimental period, with the exception of the rats on the high calcium-low phosphorus diet fed vitamin D. These 23 day-old animals are representative of the experimental animals at the time they were placed on the experimental diets. The ash weight of the femurs of the 23 day-old group is 23.4 mg.; the ash weight for the high calcium-low phosphorus group fed vitamin D is 33.18 mg.; the ashed weight of the other five groups is between 13.06 and 21.46 mg. Thus, in five of the six experimental groups decalcification takes place in the femurs. In contrast to this decalcification, there is a marked lead deposition in each of these five groups. This is a clear demonstration of the inadequacy of the generally accepted statement (11) that, "...the direction of the lead stream is similar to that of the calcium stream—that, when calcium is being deposited in the bones, circulating lead is also deposited in the bones; and when calcium is being pulled from the bones, some stored lead is also liberated." *In the above mentioned five experimental groups, conditions which caused the removal of calcium from the bones caused the deposition of lead.*

DISCUSSION

It is evident from these experiments that the influence of at least three dietary factors, namely calcium, phosphorus, and vitamin D, must be considered in the understanding of the biochemical behavior of lead. Two of these factors must be known before the effect of the third can be predicted. Vitamin D has a different effect, quantitatively, on the three diets varying in calcium and phosphorus content and ratios. Calcium has a different effect on diets which vary in phosphorus and vitamin D content. The effect of phosphorus is also different with variations of the calcium and vitamin D of the diets. The emphasis in the past has been on calcium (11–14) and later on calcium and phosphorus (15, 16). In some experiments the vitamin D has been mentioned but its effect on lead concentration in blood

and bone was not clear (17, 18). Calvery, in a recent paper, mentioned the observation that on a lead-containing rickets-producing diet the addition of vitamin D increases bone lead. However, the calcium and phosphorus content of this diet is not defined (19). Shelling (15) was the first to control these three variables. However, he did not obtain quantitative data of the lead content of blood or bone but used toxicity and growth as criteria. In preliminary experiments from this laboratory the dietary calcium, phosphorus, vitamin D, and lead content were well defined. It was therefore possible to indicate qualitatively the influence of calcium, phosphorus, and vitamin D on the lead content of blood and bone. However, since the lead content of the different diets varied, it was not possible to compare the results quantitatively. The present experiments extended the preliminary work by maintaining a constant level of lead in all the experimental diets.

The data presented in this report may be explained by postulating that lead, phosphorus, and vitamin D form a system for lead deposition analogous to the well established system of calcification for calcium (9, 10, 20–22). In such a system one may propose that there is an ideal lead to phosphorus ratio in the diet at each lead level that is most favorable for lead deposition in the bones. Either the addition or the removal of phosphorus would decrease the amount of lead deposited. Addition of any agent that behaves as if it removed phosphorus would have the same effect as direct phosphorus removal. Such an agent may be the addition of calcium. Vitamin D in this system may be expected to cause a general improvement in the deposition of lead to compensate for a disproportionate dietary ratio, and improve the deposition on any given ratio. The experimental evidence is discussed below.

It was observed that the low calcium-low phosphorus Diet A caused the greatest degree of lead deposition. This diet, therefore, was the most ideal for deposition of lead in the present series of experiments. Addition of calcium or phosphorus reduced the degree of lead deposition on this diet. The calcium was added here in such great amounts that in effect it removed very large amounts of phosphorus that might have been used for the deposition of lead. The addition of phosphorus to the low

calcium-low phosphorus diet decreased the lead to phosphorus ratio so that lead deposition was adversely affected. The addition of vitamin D increased the deposition of lead on all types of diets. These observations on the deposition of lead indicate a strong analogy to calcification.

The analogy between the deposition of lead and calcium may be carried further to include the idea that the type of dietary lead to phosphorus ratio for optimal deposition of lead is approximately similar to the type of dietary calcium to phosphorus ratio that is optimal for calcification. On this basis the optimal deposition of lead observed on the low calcium-low phosphorus diet becomes rational. In the low calcium-low phosphorus diet, the lead was 0.82 per cent and the phosphorus was 0.262 per cent, with negligible amounts of calcium (0.03 per cent). This amount of lead is the molar equivalent to 0.156 per cent calcium ($Ca:P = 0.156/0.262 = 0.6$). A dietary ratio of calcium to phosphorus of 0.6 is almost optimal for calcification at a phosphorus level of 0.262 per cent (10, 20-22). The addition of 1.00 per cent calcium or 0.6 per cent phosphorus (the amount used in our lead experiments) would markedly reduce the calcifying properties of this diet. In the light of this the optimal deposition of lead in the low calcium-low phosphorus group is understandable, since this diet appears to have almost the ideal molar ratio of metal to phosphorus for the deposition of the metal. The decrease in deposition of lead on the addition of 1.0 per cent calcium or 0.6 per cent phosphorus also becomes predictable. The objection may be raised that the effect of the addition of calcium to a calcium-containing diet on calcification is not analogous to the effect of the addition of calcium to a lead-containing diet on lead deposition. There is ample evidence, however, that the addition of metals other than calcium that are excreted as insoluble phosphates affects the process of calcification. Low phosphate rickets has been induced in rats by the addition of lead (15), strontium (23-29), magnesium (27-31), iron (32), beryllium (33-36), and manganese (37) on diets that were almost optimal for calcification in the absence of these metals. The addition of calcium to diets that are optimal from the point of view of deposition of lead may be considered in the same light as the addition of one of the above metals on calcification.

The action of vitamin D on lead deposition fits in with the proposed analogy between deposition of lead and calcium. Vitamin D increases the amount and the degree of lead deposition on all the experimental diets, in which respect it is similar to its effect on calcification on diets containing similar phosphorus levels.

The blood lead values may be explained by assuming a relationship between blood lead and dietary lead to phosphorus ratios similar to the relationship between blood calcium and dietary calcium to phosphorus ratios. As the lead to phosphorus ratio is increased, the blood lead value is increased. This means in effect that as phosphorus is added to the diet the blood lead is depressed (2), and as phosphate is removed by the addition of calcium to the diet, the blood lead values are elevated. Kowaloff (38), in a clinical study in our laboratory, has shown that the addition of phosphate to the diet of a young child suffering from lead poisoning depressed the lead of the whole blood. Furthermore, he has shown a reciprocal relationship between the lead in the whole blood and the serum phosphorus.

The influence of vitamin D on blood lead is similar to its influence on blood calcium on corresponding dietary metal to phosphorus ratios. In the low calcium-low phosphorus Diet A, the type of ratio of lead to phosphorus (as explained earlier) is similar to a calcium to phosphorus ratio of 0.6. On this type of ratio, serum calcium is elevated owing to the influence of vitamin D (9, 10). On Diet A blood lead is also increased. On the high calcium-low phosphorus Diet B, because of the removal of phosphorus by the large amounts of dietary calcium present, the effective lead to phosphorus dietary ratio is very high. On this type of disproportionate diet blood calcium is practically normal to start with. Vitamin D acts mainly on the phosphorus, having very little effect on the calcium (9, 10). It was observed that blood lead was highest in this type of diet, and vitamin D had very little effect. On the high phosphorus-low calcium Diet C, the type of lead to phosphorus ratio is similar to a calcium to phosphorus ratio of 0.18. On this type of ratio the blood calcium is low, and responds with difficulty to the action of vitamin D (9). It was observed that the lead in the blood was lowest on this diet, and the response to the action of vitamin D was not marked.

The complete explanation of blood lead in relation to dietary calcium, phosphorus, and vitamin D (as has been worked out for calcium with relation to these same factors) must await additional data. The difference between lead and calcium in the blood is that calcium is present mainly in the serum, whereas lead is present mainly in the red blood cells. Data which will carefully define the relationship of serum lead and red cell lead to each other and to the diet are necessary.

In these experiments, the *inadequacy* of the generally accepted statement (13) that "...the direction of the lead stream is similar to that of the calcium stream—that, when calcium is being deposited in the bones, circulating lead is also deposited in the bones; and when calcium is being pulled from the bones, some stored lead is also liberated" (11) is clearly demonstrated. It is shown that simultaneous lead deposition and removal of calcium from the bone is possible. It is further shown that increased calcification does not necessarily mean increased lead deposition. In the one group in which calcium deposition did take place there was less deposition of lead than in another group in which decalcification took place.

Lead deposition is directed by a system of its own, which is governed by the same laws as is calcium deposition but does not necessarily go in the same direction. The effect of calcium on the deposition of lead is essentially competitive. Calcium tends to remove phosphorus available for deposition of lead. This property of calcium may be advantageous for lead deposition in low lead-high phosphorus diets by effecting the removal of excess phosphorus. However, if too much calcium is added, phosphorus necessary for the deposition of lead will be removed and thus interfere with lead deposition. This is offered as an explanation for the contradictory results obtained in deleading experiments by the use of calcium (11, 12, 14, 39, 40).

In connection with the above picture of lead deposition, lead may be assumed to precipitate in the bone cell as some phosphate salt, similar to the precipitation of calcium as calcium phosphate. Lead phosphates and calcium phosphates are extremely insoluble and therefore their precipitation may be discussed on the basis of the solubility product principle. The solubility products of secondary and tertiary lead and calcium phosphates are given

below (see also references 41–43)), where A = activity, C = concentration, μ = ionic strength = $\frac{1}{2}(m_1 z_1^2 + m_2 z_2^2 \cdots)$, m = molarity of ion, z = valence of ion.

$$[A_{Pb^{++}}]^3[A_{PO_4^{\equiv}}]^2 = 10^{-42.00} \text{ at } 37.5°$$

$$[A_{Pb^{++}}][A_{HPO_4^{=}}] = 10^{-9.62} \text{ at } 37.5°$$

$$[C_{Ca^{++}}]^3[C_{PO_4^{\equiv}}]^2 = 10^{-27.71} \text{ at } 38°, \mu = 0.155$$

$$[C_{Ca^{++}}][C_{HPO_4^{=}}] = 10^{-6.47} \text{ at } 38°, \mu = 0.155$$

At μ = 0.16 and 37.5° the activity coefficient γ $HPO_4^{=}$ is 0.23 (41) and γ $PO_4^{\equiv}$ is 0.15. The latter was obtained by calculation of the $PO_4^{\equiv}$ ion concentration from total phosphate as in the paper of Logan and Taylor (42), since their solubility product is used for tertiary calcium phosphate, and with the same relationship between total phosphate and $PO_4^{\equiv}$ ion activity as calculated by Millet and Jowett (41). $[A_{PO_4^{\equiv}}]$ divided by $[C_{PO_4^{\equiv}}]$ is the activity coefficient.

By multiplying the $PO_4^{\equiv}$ ion concentration with the activity coefficient, one obtains $[C_{Ca^{++}}]^3 [A_{PO_4^{\equiv}}]^2 = 10^{-29.36}$.

Dividing the above equation into $[A_{Pb^{++}}]^3 [A_{PO_4^{\equiv}}]^2 = 10^{-42.00}$ one obtains $[A_{Pb^{++}}]^2/[C_{Ca^{++}}]^3 = 10^{-12.64}$ or $[A_{Pb^{++}}]/[C_{Ca^{++}}] = 10^{-4.21}$.

The activity coefficient of Pb^{++} ion is 0.17 at μ = 0.16 (41). Multiplying the activity of Pb^{++} ion by 1/0.17, one obtains the concentration of Pb^{++} ion and the above expression becomes $[C_{Pb^{++}}]/[C_{Ca^{++}}] = 10^{-3.44}$. In the blood serum at a total calcium of 2.5 mM (10 mg. per cent) the Ca^{++} ion concentration is approximately 1.1 mM (44). Therefore, if the blood serum is in equilibrium with tertiary lead and calcium phosphates, the serum Pb^{++} ion concentration should be 0.008 mg. per 100 cc. of serum. In a similar manner, the lead ion concentration should be 0.042 mg. per 100 cc. of serum if the secondary calcium and lead phosphates are in equilibrium with the blood serum. The serum lead in these experiments was higher than those calculated above and it is therefore likely that the lead in the blood serum is only partially ionized. Since the degree of ionization of lead in the blood is not known at present, it is not possible to predict total lead in the blood serum in the above manner.

In the precipitation of the bone calcium phosphate it is believed that the initial aggregation of atoms formed from solution for

precipitation is $CaHPO_4$, which changes into the crystalline $Ca_3(PO_4)_2$ (43, 45, 46). It is possible that lead phosphate is deposited in a similar manner, because the serum in these experiments appears to be more supersaturated with respect to $Pb_3(PO_4)_2$ than with respect to $PbHPO_4$. More data on the nature of the lead salt deposited and the degree of ionization of lead in blood serum are necessary before conclusions can be finally drawn.

In a solution containing calcium, lead, and phosphate ions, calcium phosphate and lead phosphate may precipitate independently or else the precipitation of one salt may cause the coprecipitation of some of the other salt. If there are two independent solids precipitating, the solubility product principle may be applied quantitatively. If there is coprecipitation, the solubility product principle can be applied only qualitatively.

If the two salts behave independently, then, at an equilibrium of the two solids with the solution, the concentration of the ions will be such that the solubility products of lead and calcium phosphates are satisfied. If calcium ions are added to such a system, calcium phosphate will precipitate and the phosphate concentration in solution will be diminished. Thus, the solubility product of lead phosphate would no longer be satisfied. In order to reach the solubility product of lead phosphate the concentration of lead ions would have to be increased, and this is attained by the solution of solid lead phosphate until equilibrium is again reestablished. Similarly, the addition of lead ions would cause the precipitation of lead phosphate and the solution of calcium phosphate. The addition of phosphate ions to the equilibrium system would cause the precipitation of both lead and calcium phosphates, whereas the removal of phosphate ions (by either a third ion such as Be^{++} or the formation of an un-ionized complex) would cause a solution of both lead and calcium phosphates. The above considerations hold true both for the tertiary and the secondary phosphates at equilibrium. Thus, if the two salts behave independently, there are four possibilities: (1) precipitation of calcium phosphate with re-solution of lead phosphate, (2) precipitation of lead phosphate with re-solution of calcium, (3) simultaneous precipitation of both, (4) simultaneous solution of both. In these experiments processes (2) and (3) took place in the bones and they are both possible from the above considera-

tions. Whether process (1) or (4) can take place will be shown in deleading experiments in progress at present.

If coprecipitation of lead salts with calcium phosphate occurs, but only to a small degree, then the above consideration will still hold. If coprecipitation is marked, then the solubility product principle cannot predict anything more than the formation of the first crystal. If lead deposition were invariably to follow the path of calcium, as claimed by some investigators (11–13), it would imply that lead deposition is coprecipitation on the bone calcium phosphate salts, and not an independent precipitation of lead phosphate. In these experiments, where simultaneous decalcification and lead precipitation took place, it is indicated that lead deposition is a precipitation of lead phosphate and not purely a coprecipitation phenomenon of calcium phosphate. (The coprecipitation hypothesis might be true if simultaneous coprecipitation of lead is assumed on new calcium phosphate precipitate and a very rapid resorption of the old calcium phosphate salt in the bone, but there is no reason at present to assume that this hypothesis is correct.)

In examination of the causes of the blood lead values obtained, the probable reactions in the gastrointestinal tract can be studied by means of the solubility product principle. Here the situation is more complex than in the blood. In the gastrointestinal tract, the pH, ionic strength, and the composition undergo wide variations, depending on the diet and gastrointestinal secretions; whereas in the blood the pH, ionic strength, and the composition are fairly constant. In the experiments described in this paper the basal diet was constant, the variations being only in the calcium, phosphorus, and vitamin D. It can be therefore assumed that the pH, ionic strength, and the composition were as constant as possible in physiological experiments, with the exception of calcium, phosphorus, and vitamin D. With the above limitations in mind, the solubility product principle can be applied to the gastrointestinal tract and a rational explanation may be offered for the blood lead values observed. In this explanation it is assumed that absorption per unit of time is proportional to the ionic concentration of lead, and the blood lead concentration follows the absorption. With these assumptions, the additions of phosphate should interfere with the absorption of lead by

decreasing the ionic concentration of lead, owing to the precipitation of lead phosphate. The addition of calcium should increase the absorption of lead by removing phosphate, thus causing an increase in the lead ion concentration by the mechanism explained earlier. Thus the increase in blood lead concentration on the addition of calcium to Diet A and the decrease of blood lead values on the addition of phosphate to Diet A become also rational.

The above considerations do not explain the rôle of vitamin D on blood lead but may be a limiting factor on the extent to which vitamin D alters the absorption and retention of lead.

SUMMARY

1. Experiments were designed to study the influence of dietary calcium, phosphorus, and vitamin D on the biochemical behavior of lead in young rats. The diets used varied with respect to these three factors but were similar in their lead content.

2. The results show that influence of all three dietary factors, namely calcium, phosphorus, and vitamin D, must be considered in the biochemical behavior of lead.

3. Lead deposition in the bones may be explained by postulating that lead, phosphorus, and vitamin D form a system of lead deposition analogous to the system of calcification. The addition of calcium tends to remove phosphorus from the system for lead deposition.

4. Blood lead values obtained may be explained by assuming a relationship between blood lead and dietary lead to phosphorus ratios similar to the relationship between blood calcium and dietary calcium to phosphorus ratios. The influence of vitamin D on blood lead is similar to its influence on blood calcium on corresponding dietary metal to phosphorus ratios.

5. It was experimentally demonstrated that simultaneous lead deposition and removal of calcium from the bones is possible. Furthermore, it was shown that increased calcification does not necessarily mean increased lead deposition.

6. It is concluded therefore that lead deposition is directed by a system of its own, which is governed by the same laws as is calcium deposition but does not necessarily go in the same direction. The effect of calcium on the deposition of lead is essentially

competitive, because it tends to remove phosphorus available for lead deposition.

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THE CATALYTIC HYDRATION OF CARBON DIOXIDE

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During a study of the effect of oxidation and reduction upon the activity of carbonic anhydrase, it was found that bromine has, of itself, a strong catalytic effect upon the hydration of CO_2. This observation led to the systematic study of the catalytic effect of the halogens and halogen compounds herewith presented. Recently, Roughton and Booth (1938) published a comprehensive study of the catalytic effect of buffers upon the hydration of CO_2. Among a number of salts investigated, they found that sulfite and selenite were the strongest catalysts. We shall present data showing that bromine and hypobromous acid exceed sulfite and selenite in their catalytic activity.

Measurements of the uncatalyzed hydration were made by Faurholt (1924), by Brinkman, Margaria, and Roughton (1933), and by Stadie and O'Brien (1933), using different methods. Though their results differ but slightly among themselves, they are not entirely comparable, as it is now known that buffer salts, which are necessarily present, have, of themselves, a catalytic effect. The measurements to be reported in this paper permit the estimation of the rate of hydration at different pH values when the buffer concentration equals zero.

Methods

The hydration of CO_2 was measured manometrically in a manner similar to that of Brinkman, Margaria, and Roughton (1933) (Fig. 1). As the reaction chamber, a boat-shaped vessel, *6*, with a ground joint was used. (It was connected with the manometric system by means of copper tubing and a 3-way stop-cock, *5*.) The manometer system consisted of the closed manometer of a

Van Slyke gas analysis apparatus and a volumetric Van Slyke gas burette. One of the upper outlets of the gas burette was connected with a Kipp generator as a source of CO_2; to the other outlet, a 3-way stop-cock was sealed, *5*. One outlet of this stop-cock was connected with the copper tubing leading to the reaction vessel; another outlet was connected to a vacuum pump. The

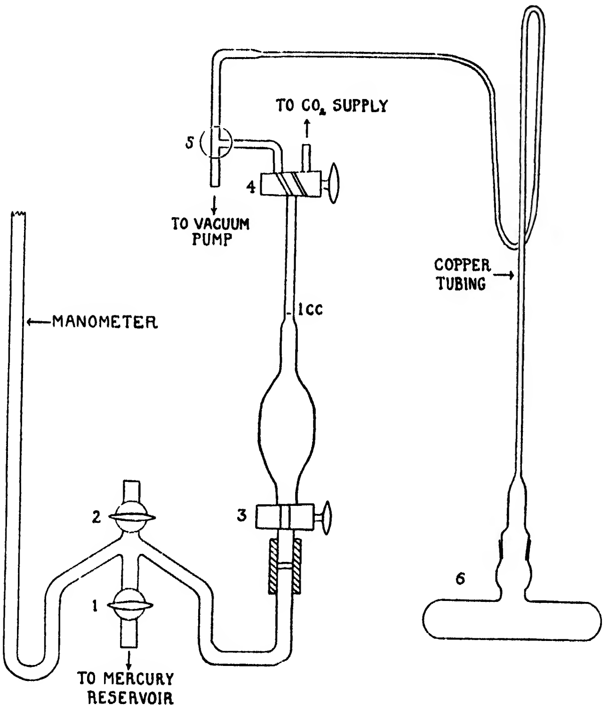

FIG. 1. Apparatus used to determine the rate of hydration of CO_2. For a description of the apparatus and its use, see the text.

reaction vessel was attached to a shaker, which ran with a speed of 480 to 500 oscillations per minute.

EXPERIMENTAL

Procedure—A measured amount of buffer solution was placed in the reaction vessel and the vessel connected with the copper tubing at *6*. The whole system, vessel and burette, was then evacuated to the vapor pressure of the buffer solution. The

reaction vessel was shaken during the evacuation to extract all gases. Stop-cock *5* was then closed, and CO_2 was drawn into the burette through stop-cock *4* and measured. Stop-cock *4* was then turned so that the burette and reaction vessel were connected, the CO_2 thereby passing into the reaction vessel. The mercury was brought to the 1.0 cc. mark of the burette. During the reaction, the mercury could be brought back to the 1.0 cc. mark by manipulating stop-cock *1*. (Stop-cock *2* was not used save for the ejection of gas.) For pressure readings, stop-cocks *1* and *3* were closed and the manometer reading thus fixed at any definite time. In this way, accurate pressure measurements at constant volume were possible.

In our experiments, the liquid phase was 10 ml., the gas phase 41.8 ml. All experiments were carried out at a temperature of 5.0°. The whole apparatus, all solutions, and the gas generator were kept in a room at that temperature. The buffer solutions employed were 0.2 M phosphate, 0.1 M pyrophosphate, 0.1 M borate, and 0.1 M carbonate. In each experiment, the pH of the buffers was measured electrometrically with the glass electrode.

In most experiments, an initial CO_2 pressure of 100 mm. of Hg was used. Several series of experiments were performed at lower and higher pressures with similar results. It was found that when the initial CO_2 pressure was 100 mm. of Hg, the corresponding equilibrium pressure, following the physical solution of CO_2 but before hydration began, was 79 mm.

Most of the experiments were carried out on the hydration of CO_2, only a few on the dehydration. It was found that, under comparable conditions, the catalyzed and uncatalyzed hydration and dehydration reached the same equilibrium state. This may be interpreted as evidence that the catalytic effect was the same in both the hydration and dehydration reactions.

Calculation of Velocity Constants and Catalytic Effect—The hydration of CO_2 proceeds at pH $\gtreqless 7$ according to the reaction, $CO_2 + H_2O \rightleftharpoons H_2CO_3$. The hydration rate may be expressed as $dCO_2/dt = k_{CO_2}[CO_2] - k_{H_2CO_3}[H_2CO_3]$ where k_{CO_2} is the velocity constant for the hydration of CO_2 and $k_{H_2CO_3}$, the constant for the dehydration of H_2CO_3, respectively. At the beginning of the hydration, the opposing reaction (dehydration) can be neglected in calculation of the hydration velocity constant, k_{CO_2}.

The derivation of the equations employed is given in the paper by Brinkman, Margaria, and Roughton (1933), and need not be repeated here. The symbols used and their definitions follow. a = concentration of CO_2 at time 0; x = concentration of CO_2 at time t; P_0 = pressure of CO_2 at time 0; P = pressure of CO_2 at time t; V_G = volume of gas phase; V_L = volume of liquid phase; α = absorption coefficient of CO_2 (Bunsen). The time is measured in seconds.

The equation for k_{CO_2} in the above terminology is as follows:

$$k_{CO_2} = \frac{1}{t_2 - t_1} \times \frac{\left(\frac{V_G}{V_L} + \alpha\right)}{\alpha} \times \ln \frac{P_1}{P_2} \quad (1)$$

In solutions whose pH exceeds 7, the reaction $CO_2 + OH^- \rightleftharpoons HCO_3^-$ must be taken into account. In very alkaline solutions (above pH 10), the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ can be neglected. Then $-dx/dt = k_{CO_2,OH^-} \cdot [CO_2]\,[OH^-]$ where k_{CO_2,OH^-} is the velocity constant of the reaction between CO_2 and OH^- (the back reaction being neglected).

By analogy with Equation 1

$$k_{CO_2,OH^-} \times [OH^-] = \frac{1}{t_2 - t_1} \times \frac{\left(\frac{V_G}{V_L} + \alpha\right)}{\alpha} \times \ln \frac{P_1}{P_2} \quad (2)$$

Between pH 7 and 10, the over-all constant, k, for the two reactions occurring simultaneously is

$$k = k_{CO_2} + k_{CO_2,OH^-} \times [OH^-] = \frac{1}{t_2 - t_1} \times \frac{\left(\frac{V_G}{V_L} + \alpha\right)}{\alpha} \ln \frac{P_1}{P_2} \quad (3)$$

In addition to the calculation of the hydration velocity constant, the half time of the completion of the hydration was estimated in all experiments. If the amount and concentration of buffer and the initial CO_2 pressure are kept constant, the half time depends only upon the two velocity constants. As a measure of the relative catalytic effect of different substances, the concentrations of catalysts which decreased the half time of the reaction by 50 per cent were compared. This is equivalent to determining what

concentration of catalyst is necessary to double the hydration and dehydration velocity constants.

Calculations of the velocity constants are based on the assumption that the reaction is of the first order. The velocity constant does not vary significantly if the CO_2 concentration is changed over a rather wide range (Table I). These results would seem to justify the treatment of the reaction as of the first order.

Uncatalyzed Hydration of CO_2

Faurholt (1924), Brinkman, Margaria, and Roughton (1933), and Stadie and O'Brien (1933) have investigated the kinetics of the hydration of CO_2. The velocity constants found by these

TABLE I

Hydration of CO_2 in 0.2 M Phosphate at pH 7.0 at Varying Initial Pressures

The pressure is that of CO_2 at the beginning when CO_2 is only in the gas phase.

Initial pressure of CO_2	k_{CO_2}
mm. Hg	
50	0.007
100	0.0075
250	0.0072
500	0.0077
1100	0.0076

authors, and expressed in the same units, were $k_{CO_2} = 0.003$ and $k_{CO_2,OH^-} = 930$ at 0° (Faurholt); $k_{CO_2} = 0.0026$ and $k_{CO_2,OH^-} = 840$ at 0° (Brinkman *et al.*); $k_{CO_2} = 0.0027$ at 0° (Stadie and O'Brien).

These studies were carried out before it was discovered by Roughton and Booth (1938) that the presence of buffer salts modifies the velocity of the hydration of CO_2. Our measurements in buffer solutions of different concentrations confirm their findings. The measurement of the velocity of hydration in buffer solutions of varying strength and at constant pH allows the extrapolation of the velocity constant to zero buffer concentration.

The results of experiments with phosphate buffer and at pH 6.15, 7.0, and 7.55 are shown in Fig. 2. It is seen that a linear relation exists between the value of the hydration constant, k_{CO_2}, and the molar concentration of the buffer salt. From these data,

the hydration constant of CO_2 at zero salt concentration and 5° is found by extrapolation to have the following values: k_{CO_2} = 0.0036 at pH 6.15, 0.0037 at pH 7.0, 0.0039 at pH 7.55. Using the values of k_{CO_2} reported at 18° and 0° by Faurholt and by Brinkman, Margaria, and Roughton and applying the Arrhenius equation to our data at pH 7.0 yield the value k_{CO_2} = 0.0020 at 0°

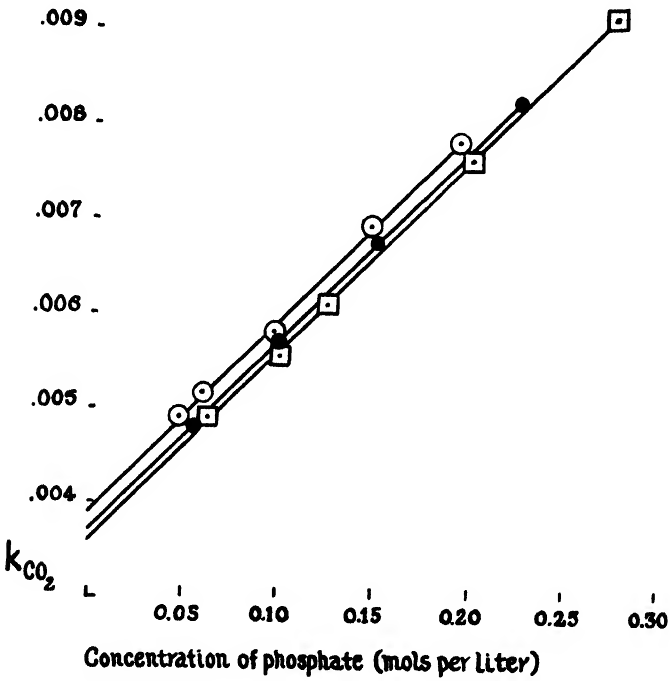

FIG. 2. Velocity constant of hydration of CO_2, k_{CO_2}, at different pH values and different phosphate concentrations. Determinations at pH 7.55 are designated as ⊙, at pH 7.0 as ●, at pH 6.15 as ⊡.

and zero salt concentration. This compares favorably with the value k_{CO_2} = 0.0021 reported by Roughton and Booth.

The results of measurements of the hydration rate at 5° in buffer solutions whose pH varied between 8.1 and 10.1 are given in Fig. 3. It is seen that the over-all constant k varies linearly with the concentration of the buffer. Although the catalytic effect of borate buffer at pH 9 is very different from that of pyrophos-

phate at the same pH, it is of interest that both values of k extrapolated to zero buffer concentration are in good agreement.

From these data and Equation 3, the values of k_{CO_2,OH^-} at 5° have been calculated, assuming $k_{CO_2} = 0.0037$ and $pK_w = 14.73$ (Harned and Hamer, 1933). The error of the graphical method

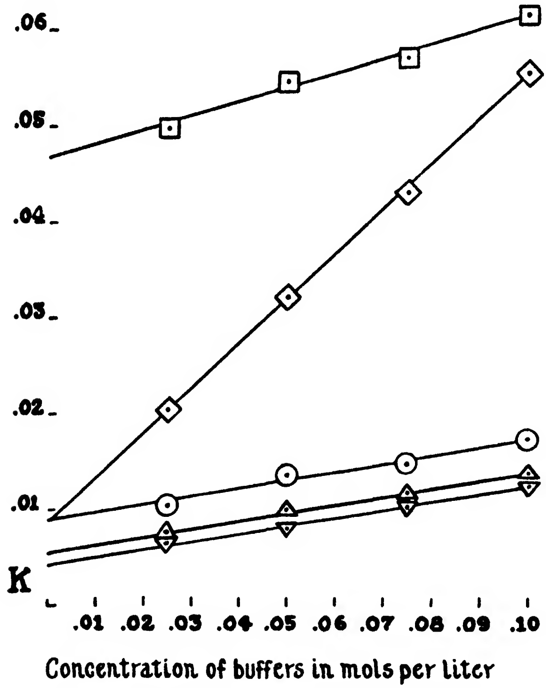

FIG. 3. Velocity constant of hydration of CO_2 ($k = k_{CO_2} + k_{CO_2 \cdot OH^-} \times [OH^-]$) at different pH values and different buffer concentrations. Determinations in carbonate buffer at pH 10.1 are designated as ⊡; in borate buffer at pH 9 as ◇; in pyrophosphate buffer at pH 9 as ⊙, at pH 8.55 as △, at pH 8.1 as ▽.

of extrapolation to zero salt concentration is approximately ±0.002 at pH 8.1, 8.55, and 9.0; it is ±0.0005 at pH 10.1. The effect of these errors on the estimated values of k_{CO_2,OH^-} has been indicated in in Table II. Between pH 8.1 and 9, the value of k_{CO_2,OH^-} is approximately 2900, although there is considerable uncertainty about its exact magnitude. This is due to the fact

that k_{CO_2} makes up a large proportion of k. At pH 10.1, the value of k_{CO_2,OH^-} was 1840.

Corrected to 0°, $k_{CO_2,OH^-} = 1200$ (with the temperature coefficient of the reaction from the data of Faurholt), or $k_{CO_2,OH^-} = 1400$ (with the temperature coefficient of Brinkman, Margaria, and Roughton). It should be pointed out that our lowest estimate for the value of k_{CO_2,OH^-} at pH 10.1 is considerably higher than those previously reported (Faurholt, $k_{CO_2,OH^-} = 930$; Brinkman, Margaria, and Roughton, $k_{CO_2,OH^-} = 840$). We have no explanation to offer which would account at present for this difference in results.

Following the ideas of Thiel (1913), Thiel and Strohecker (1914), and Faurholt (1924), we have assumed that the increase of the hydration rate in alkaline solution is due to the reaction between CO_2 and OH^-. The experimental data satisfy the treatment of the hydration as a reaction of CO_2 with H_2O and with OH^-. However, the increase of the hydration rate in alkaline solutions could equally well be attributed to catalysis by hydroxyl ions.

TABLE II

Velocity Constants of Hydration of CO_2 in Alkaline Solutions According to Reaction, $CO_2 + OH^- \rightleftharpoons HCO_3^-$, at Zero Buffer Concentration

pH	Buffer	Value of k at 0.0 buffer concentration	k_{CO_2,OH^-}
8.1	Pyrophosphate	0 0044 ± 0.0002	2950 ± 850
8.55	"	0.0056 ± 0 0002	2900 ± 300
9.0	"	0.0090 ± 0.0002	2850 ± 100
9.0	Borate	0 0090 ± 0.0002	2850 ± 100
10.1	Carbonate	0.0468 ± 0.0005	1840 ± 25

Catalytic Effect of Halogens

Materials Used—The catalytic effects of chlorine, bromine, and iodine, and a number of halogen compounds, have been studied. The reagent chemicals, bromine, iodine, chloride, bromide, iodide, chlorate, bromate, iodate, perchlorate, and periodate were used without further purification. Chlorine was prepared by oxidation of HCl by manganese dioxide. Hypochlorous, hypoiodous, and hypobromous acids were always freshly

prepared by the reaction between the halogens and mercuric oxide. Hypochlorous and hypobromous acids were purified by vacuum distillation in the absence of light and were kept at low temperature. An impure hypoiodous acid was prepared by adding iodine in water to mercuric oxide and filtering through a glass filter. Further purification of the hypoiodous acid was not attempted because of its rapid decomposition. The strength of these acids was determined both by iodometric titration and by titration with standard alkali. Since extremely low concentrations of hypochlorite and hypobromite were used in the catalysis experiments, the necessary amounts of acid were added to the buffer solutions without previous neutralization.

It was important, in our experiments, to know the dissociation constants of hypochlorous and hypobromous acid. Values given for the dissociation constant of hypochlorous acid, $K = [H^+][OCl^-]/[HOCl]$, are 3.7×10^{-8} at 17° (Sand, 1904), 0.067×10^{-8} at 25° (Noyes and Wilson, 1922), 1.0×10^{-8} at 25° (Soper, 1924), 5.1×10^{-8} at 30° (Giordani, 1924), and 4.5×10^{-8} (Skrabal, 1938). We have determined the dissociation constant at 5° by titration of a 0.06 M solution of HOCl with sodium hydroxide, measuring the pH electrometrically with a glass electrode, and found $K' = 2.8 \times 10^{-8}$, or pK' = 7.55.

The dissociation constant of hypobromous acid has recently been determined at 20° by Shilov and Gladtchikova (1938) and found to be 2.06×10^{-9}. We have redetermined this constant at 5° and obtained the value 1×10^{-9}, pK' = 9.0 (Kiese and Hastings, 1939).

As stated earlier, the catalytic effect was measured by the concentration of the catalyst which was necessary to double the rate of hydration.

Bromine and Hypobromous Acid

The effect of bromine upon the hydration of CO_2 was tested over the pH range 6.1 to 10.1. The results are given in Table III. Bromine catalyzed the hydration over the whole range studied, the effect increasing with increasing pH, up to pH 9. The catalytic effect of hypobromous acid slightly exceeded that of bromine.

Bromine reacts in water according to the reaction $Br_2 + H_2O =$

HBr + HOBr. The hydrolysis constant is given by the equation $[H^+] [Br^-] [HOBr]/[Br_2] = K$, where $K = 0.69 \times 10^{-9}$ at 0°, and $K = 1.76 \times 10^{-9}$ at 10° (Liebhafsky, 1934). The concentration of HOBr in a solution of Br_2 in water at pH 7.5 and less is, according to the hydrolysis constant, very low and much too small to account for the catalytic effect of these solutions. The catalytic effect is, therefore, to be ascribed to the Br_2 itself.

Chlorine and Hypochlorous Acid

Chlorine and hypochlorous acid catalyze the hydration of CO_2 similarly to bromine and hypobromous acid. Their catalytic activity, however, is only about one-tenth that of bromine. Again, Cl_2 and HOCl are roughly of approximately equal catalytic activity (Table III).

TABLE III

Catalytic Effect of Br_2, HOBr, Cl_2, and HOCl at Different pH Values

pH	Buffer	Concentration required to double rate of hydration, mM per liter			
		Br_2	HOBr	Cl_2	HOCl
6.15	Phosphate	0.44			4.4
6.90	"	0.19	0.17	3.0	2.75
7.55	"	0.09	0.05	0.9	0.7
9.0	Borate	0.02	0.01	0.32	0.27
10.0	Carbonate	0.03	0.02	0.3	0.27

The proportion of Cl_2 present as HOCl in a solution of Cl_2 is much larger than in the case of bromine. From measurements of Jakowkin (1899) at 25°, the hydrolysis constant of Cl_2 is calculated to be $K = 4.48 \times 10^{-4}$. If the temperature coefficient is similar to that found for bromine, the hydrolysis constant will be approximately 1×10^{-4} at 5°. Therefore, over the pH range covered in our experiments, the proportion of the chlorine present as Cl_2 was small and the concentration of HOCl rather high. It is, therefore, not possible to estimate separately the relative catalytic effects of Cl_2 and of HOCl.

The question of whether the effect of pH on the catalytic effect of Br_2, HOBr, Cl_2, and HOCl could be attributed to the concentration of OBr^- and OCl^- ions has been considered. By compar-

ing the relative concentrations of these ionic species with their corresponding catalytic effects, we have found that such an explanation is inadequate.

The close similarity in the change in catalytic activity of Br_2, HOBr, Cl_2, and HOCl with pH could be accounted for as follows: The rate of hydration of CO_2 according to the reaction, $CO_2 + OH^- \rightleftharpoons HCO_3^-$, is determined by the concentration of hydroxyl ions, other things being equal. Perhaps bromine and chlorine catalyze only this reaction and not the reaction, $H_2O + CO_2 = H_2CO_3$. This would adequately account for the observed relation between catalytic activity and hydroxyl ion concentration.

Other Halogen Compounds

Iodine and hypoiodous acid did not have any perceptible catalytic effect upon the hydration. The halogenides, halogenates, and perhalogenates have been tested in concentrations up to 0.05 and 0.08 M, and found to have a negligible catalytic effect. The catalytic effects of chloride, iodide, and fluoride reported by Roughton and Booth (1938) are of a much lower order of magnitude than those of Cl_2 and Br_2.

Carbon tetrachloride in saturated solution in 0.2 M phosphate at pH 7 did not affect the hydration rate.

Sulfite and Selenite

Sulfite and selenite were shown by Roughton and Booth (1938) to be relatively strong catalysts of the hydration of CO_2. Our results confirm these findings. From our data, which are given in Table IV, it cannot be decided which one is the stronger catalyst. (Sulfite was more active at pH 6.5, 7.0, and 9.0; selenite more active at pH 7.55 and 10.1.) Roughton and Booth found selenite more active than sulfite. Both salts are much weaker catalysts than Br_2 and Cl_2, and the relation of catalytic activity to hydrogen ion concentration is quite different from that which was found for Br_2 and Cl_2. Sulfite and selenite appear to exhibit a maximum activity between pH 7.5 and 9, which was not apparent in the case of Br_2, HOBr, Cl_2, and HOCl. The second dissociation constants of sulfurous acid, pK′ = 7.0, and selenious acid, pK′ = 8.2, were determined by electrometric titration at 5°. It seems

doubtful that the dissociation of the acids plays a determining rôle in the activity of these two catalysts.

A point of additional interest is that, although sulfide is the strongest inhibitor of carbonic anhydrase, sulfide did not inhibit the catalytic effect of sulfite.

TABLE IV

Catalytic Effect of Sulfite and Selenite at Different pH Values

Sulfurous acid, pK = 7.0; selenious acid, pK = 8.2.

pH	Buffer	Concentration required to double rate of hydration, mM per liter	
		Sulfite	Selenite
6.15	Phosphate	5.5	7.5
7.00	"	3.3	5.3
7.55	"	2.0	1.3
9.00	Borate	1.4	1.6
10.1	Carbonate	8.7	7.0

TABLE V

Comparison of Effect of Different Catalysts on Rate of Hydration and Dehydration

The half times of the reactions are given as a measure of their rates. 10 ml. of phosphate, 0.2 M, at pH 6.9.

Catalyst	Concentration	Initial CO_2 pressure	Half time of hydration	CO_2 pressure at equilibrium	Half time of dehydration
		mm. Hg	*sec.*	*mm. Hg*	*sec.*
0		227.5	122	103.0	118
Anhydrase	32 γ %	227.0	25	103.0	26
Br_2	0.72 mM per l.	227.0	31	103.5	30
HOBr	0.85 " " "	227.5	20	103.5	21
Sulfite	8.5 " " "	227.0	36	103.0	35

Comparison with Carbonic Anhydrase

The relative catalytic activity of carbonic anhydrase and the catalytically active inorganic substances have been compared. The enzyme is much stronger than any of the inorganic catalysts mentioned above. Although the enzyme preparations used in our experiments were not pure, a concentration of 1 γ per cent of our best preparations was sufficient to double the rate of hydration

at pH 7.55. (Carbonic anhydrase has its optimal activity close to pH 8; see the following paper (Kiese and Hastings, 1940).) Bromine at pH 7.55, must be present in a concentration of 0.09 mM per liter to produce the same catalytic effect. This means that weight for weight our carbonic anhydrase was more than 1000 times more active than bromine.

The question of whether the catalysts catalyze hydration and dehydration equally is of some interest. Measurements of the rates of hydration and dehydration, made under similar conditions and at the same pH, are reported in Table V. After the hydration had reached equilibrium, the shaker was stopped. The CO_2 in the reaction vessel was then quickly pumped off and the dehydration started. As pointed out above, an unequal increase of the hydration and dehydration rates would lead to an equilibrium different from the one reached by the uncatalyzed reaction. These data show that the enzyme, as well as the other catalysts, increases the rates of hydration and dehydration equally. This is shown both by the agreement between the direct measurements of these rates and from the identical positions of the equilibrium pressures.

Miscellaneous Experiments

In experiments on the thermal decomposition of bicarbonates, Stumper (1931) found that this process can be catalyzed by a number of substances. The most powerful catalysts were some kinds of charcoal, graphite, and tungstic oxide. Since the decomposition of bicarbonate involves the two reactions $2HCO_3^- \rightarrow CO_3^{=} + H_2CO_3$ and $H_2CO_3 \rightarrow CO_2 + H_2O$, we tested charcoal, graphite, and tungstic oxide for their catalytic effect upon the hydration of CO_2. The results were negative.

Platinum and palladium deposited on asbestos and colloidal palladium prepared after Pal were also found to be without effect on the hydration of CO_2.

On the other hand, hydrogen peroxide was found to double the speed of hydration of CO_2 in a concentration of 0.05 M at pH 6.9. This catalytic effect has not been further investigated.

SUMMARY

The velocity constants for the hydration of CO_2 according to the reactions, $H_2O + CO_2 \rightleftharpoons H_2CO_3$ and $CO_2 + OH^- \rightleftharpoons HCO_3^-$, were measured by a manometric method.

Bromine, hypobromous acid, chlorine, and hypochlorous acid are strong inorganic catalysts for the hydration of CO_2. Br_2 is stronger than Cl_2. They are particularly effective in the alkaline range.

Iodine, hypoiodous acid, the halogenides, halogenates, and perhalogenates do not have significant catalytic effects.

Sulfite and selenite are weaker catalysts for the hydration of CO_2 than Cl_2 and Br_2. They have an optimal activity between pH 7 and 8.

Weight for weight, carbonic anhydrase is at least 10^3 times more active than Br_2.

The dissociation constant of hypochlorous acid at 5° was found to be $K' = 2.8 \times 10^{-8}$, and that of hypobromous acid at 5°, $K' = 1.0 \times 10^{-9}$.

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FACTORS AFFECTING THE ACTIVITY OF CARBONIC ANHYDRASE

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The activity of several hydrolytic enzymes, *e.g.* papain, urease, phosphatase, can be altered by reducing or oxidizing substances. Some observations suggest that the effects of oxidizing and reducing agents on the hydrolytic and synthetic activities of the enzymes are different. For example, Waldschmidt-Leitz, Scharikova, and Schäffner (1933) report that the hydrolysis of glycerophosphate by kidney phosphatase is inhibited by sulfide and by cysteine, although these substances do not change the synthetic activity of the enzyme. Kayashima (1938) reports that the hydrolytic activity of liver esterase is increased by reduction and decreased by oxidation, while the synthetic activity is increased by oxidation and decreased by reduction. Lens protein and succinic dehydrogenase were used to reduce the enzyme and aeration in the presence of copper sulfate to oxidize it. These observations are not consistent with the classical definition of a catalyst.

In undertaking a study of the modification of the lytic and synthetic properties of an enzyme, it seemed desirable to choose one in which both properties could be studied with the same degree of precision. Carbonic anhydrase meets these requirements admirably because both the hydration of CO_2 and the dehydration of H_2CO_3 can be accurately measured.

The experiments to be presented herein are concerned with (1) the further purification of the enzyme, carbonic anhydrase, (2) the effect of various oxidizing and reducing agents on the enzyme, (3) the effect of pH, and (4) the reexamination of the effect of other substances reported to be inhibiting agents, such as CO, cyanide, and sulfide (Meldrum and Roughton, 1934).

Since the papers of Meldrum and Roughton (1934) and Stadie and O'Brien (1933) deal extensively with the properties of carbonic anhydrase, they will not be reviewed again here.

Measurement of Activity of Enzyme—The activity of the enzyme was measured by the effect on the rate of hydration of CO_2 and dehydration of H_2CO_3 in 0.2 M phosphate buffer solution, pH 7.0. Hydration and dehydration rates were measured manometrically with an apparatus which is described in the preceding paper (Kiese and Hastings, 1940). Initial CO_2 pressures from 100 to 250 mm. of Hg were used. Hydration and dehydration were usually studied consecutively on the same solution. After the hydration had come to equilibrium, the shaking of the reaction vessel was stopped, the gas space rapidly evacuated, and after the shaker was started again, the rate of dehydration was measured. In many experiments, the rate of dehydration was not separately measured, but only the rate of hydration and the equilibrium pressure. From these data, any effect upon the rate of dehydration could be detected. Since the amount and concentration of buffer and the initial CO_2 pressure were kept constant, the equilibrium pressure depended upon the ratio of the velocity of the hydration and dehydration. Therefore, if a catalyst increased the speed of hydration and the reaction came to the same equilibrium as the uncatalyzed reaction, the dehydration must have been influenced by the catalyst to the same extent as the hydration.

The concentration of the enzyme which increased the reaction rate by 100 per cent was chosen as the measure of catalytic activity. The half time to the completion of the reaction was usually used as the measure of the reaction rate. The procedure followed is apparent from inspection of the curves given in Figs. 2, 3, and 5. The equilibrium pressure corresponding to zero time was found by extrapolation. All experiments were carried out at 5.0°, and were repeated two or more times.

Preparation of Carbonic Anhydrase

The enzyme was prepared from washed beef erythrocytes. In the first step, the removal of hemoglobin from the solution of hemolyzed cells, the procedure of Meldrum and Roughton and of Stadie and O'Brien was followed. To the washed erythro-

cytes were added water, alcohol, and chloroform, each in the amounts equal to half the erythrocyte volume. The mixture was shaken for some minutes, and centrifuged after several hours. The supernatant fluid containing the enzyme was then dialyzed against running water to remove the alcohol and chloroform. A repeated fractional precipitation of the solution with ammonium sulfate was then carried out. From a 1 per cent solution of the crude material, the highest concentration of enzyme was precipitated between 70 and 85 per cent saturation with ammonium sulfate. The final fraction was electrodialyzed. The whole procedure was carried out at 5°. The enzyme prepared in this way will be referred to as Preparation A. 0.35 γ of this preparation in 10 ml. of phosphate, pH 7.35, increased the speed of hydration by 100 per cent. The activity of this enzyme preparation may be compared with that of Meldrum and Roughton. Their preparation doubled the dehydration rate, at 15°, in a concentration (by weight) of 1:7,000,000 while our Preparation A had the same activity at 5° in a concentration of 1:30,000,000.

The enzyme was purified further by a procedure which we shall call fractional denaturation. The enzyme was dissolved in a slightly acid buffer and sodium bicarbonate added. A vigorous foaming ensued which denatured and precipitated protein. If the amount of bicarbonate was too large, practically all the protein in solution was denatured, including the enzyme. But if the foaming was not excessive and the protein precipitation incomplete, the enzyme remaining in solution was in a relatively purer state. The optimal amount of bicarbonate had to be determined by preliminary experiment. The enzyme so obtained was still slightly yellowish in color, but less so than before treatment. The colored substance could be adsorbed on an old aluminum hydroxide B (Willstätter and Kraut, 1924) without loss of enzyme. Continuing these two procedures, fractional denaturation and adsorption, gave a colorless enzyme preparation whose purity was increased 3 to 5 times, compared with that of Preparation A.

Some Chemical Properties of the Enzyme

The enzyme lost very little activity if kept in 0.5 per cent solution at 3° for several weeks. However, in 0.001 per cent solution at 3°, the activity decreased 50 per cent in 3 to 6 days.

The iron content of several preparations was estimated by the method of Willstätter (1920). After a single ammonium sulfate precipitation, the enzyme preparations contained 0.02 to 0.05 per cent iron. With repeated fractional precipitation with ammonium sulfate, the iron content decreased. Preparation A contained 0.008 per cent iron. Further purification did not further decrease the concentration of iron.

Since this work was completed, Keilin and Mann (1939) have reported the important observation that the activity of their carbonic anhydrase preparations is paralleled by the zinc content of the preparations, their best material containing 0.31 to 0.34 per cent zinc. Iron, copper, manganese, and magnesium were absent from their purest preparations. Unfortunately, no observations were made on the zinc content of our preparations.

No phosphorus was detected in our enzyme preparations. In view of the error of the method employed and the amount of material used, this would indicate that the phosphorus content was less than 0.01 per cent.

The mobility of the enzyme in the Tiselius cataphoresis apparatus (1937) has been determined in several experiments.[1] (Purification of the enzyme by this procedure has, so far, been unsuccessful.) The enzyme preparation was dissolved in acetate buffer at 0.1 ionic strength, concentration approximately 1 per cent. In solutions of pH 3.7, 4.3, 4.5, and 4.7, only one strong boundary was found to migrate symmetrically to the cathode. The enzyme activity always accompanied this boundary. In a solution of pH 5.3, the protein solution no longer appeared to be homogeneous. One boundary appeared to move to the cathode and another one to the anode. Both fractions contained active enzyme material. From the speed of migration of the protein in solutions of varying pH, the isoelectric point of the enzyme in acetate buffer was estimated to be approximately pH 5.

Effect of Oxidation and Reduction

It was found that a number of oxidizing agents inhibit the activity of carbonic anhydrase. In Table I, the concentrations

[1] The experiments were carried out in the Department of Physical Chemistry, Harvard Medical School, through the courtesy of Dr. Edwin J. Cohn and Dr. Ronald M. Ferry.

of the oxidizing agents which inhibited activity of the enzyme by 50 per cent are given. Some relation between oxidizing intensity and effectiveness as inhibitors is evident. Perchlorate and periodate, which are stronger oxidizing agents than chlorate, were stronger inhibitors. Chlorate was stronger than bromate,

TABLE I

Inhibition of Carbonic Anhydrase by Different Oxidizing Agents

The enzyme was dissolved in 0.2 M phosphate, pH 7.0.

Substance added	Concentration which inhibited enzyme activity 50 per cent
	mole per l.
Permanganate	5×10^{-5}
Iodine	5×10^{-5}
Periodate	1×10^{-3}
Perchlorate	2×10^{-3}
Chlorate	4.5×10^{-3}
Persulfate	1×10^{-2}
Bromate	2×10^{-2}

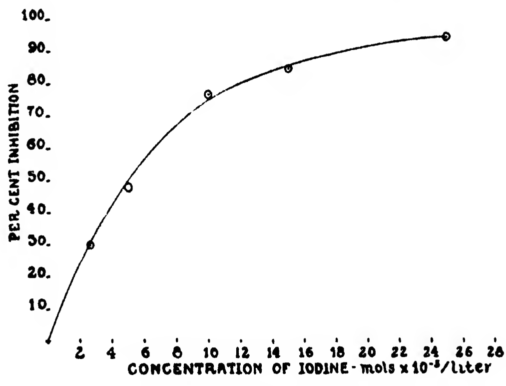

FIG. 1. The inhibition of carbonic anhydrase by iodine. Enzyme Preparation A in 0.2 M phosphate buffer, pH 7.0.

and iodate, 0.02 mole per liter, did not show any inhibitory effect. The inhibition produced by different concentrations of iodine, one of the strongest inhibitors, is shown in Fig. 1. In all experiments, a concentration of enzyme Preparation A, which, in the absence of inhibitors, increased the rate of hydration 4 times, was used. Control experiments with oxidizing agents but without

enzyme had no influence upon the reaction. In all cases, the inhibition was found to be reversible by certain reducing agents when the contact between the oxidizing agent and the enzyme did not exceed 10 minutes. Reducing agents such as ascorbic acid, cysteine, or hydroquinone, restored the activity of the enzyme completely after inhibition by the oxidizing agent. When ascorbic acid was used, pH change was avoided by addition of an equivalent amount of alkali. The pH was checked with the glass electrode. In the concentrations used, ascorbic acid did not, of itself, affect the hydration of CO_2 to a measurable extent.

In Fig. 2, a typical example of the reversible inhibition by iodine is given. The curves of comparable experiments with permanganate are essentially the same as those presented for iodine.

Several experiments were carried out with each oxidizing and reducing agent studied. The results of all comparable experiments were identical. The activity of the enzyme could be only partially restored after 2 hours contact with $KMnO_4$. With iodine, however, the inactivation was completely reversible even after 4 hours. Potassium ferricyanide, 0.01 M, did not inhibit the enzyme; nor did porphyrindin, a dye with a rather high oxidation-reduction potential (E'_0 = +0.57 volt at pH 7.0) (Kuhn and Desnuelle, 1938), inhibit the enzyme in a concentration of 0.002 mole per liter.

Unfortunately, the effect of oxygen and hydrogen with Pt or Pd as catalyst on the activity of the enzyme could not be studied, as was done with phosphatase (Kiese and Hastings, 1938). Carbonic anhydrase was completely inactive in the presence of platinized or palladium asbestos, or colloidal palladium. Apparently, the enzyme was adsorbed on the catalysts, since filtrates of solutions to which these catalysts were added were inactive.

From the data in Fig. 2, it can be seen that in both the active and inactive state, the influence of the enzyme on hydration and on dehydration was the same. This was also true for incomplete inhibition of the enzyme by different concentrations of oxidizing agent, as illustrated in Fig. 3, when the enzyme was inhibited by different concentrations of permanganate. The hydration rate and the equilibrium pressure were measured. Within the limits of accuracy of the method, the same equilibrium was reached in the catalyzed and uncatalyzed reaction. It may also be men-

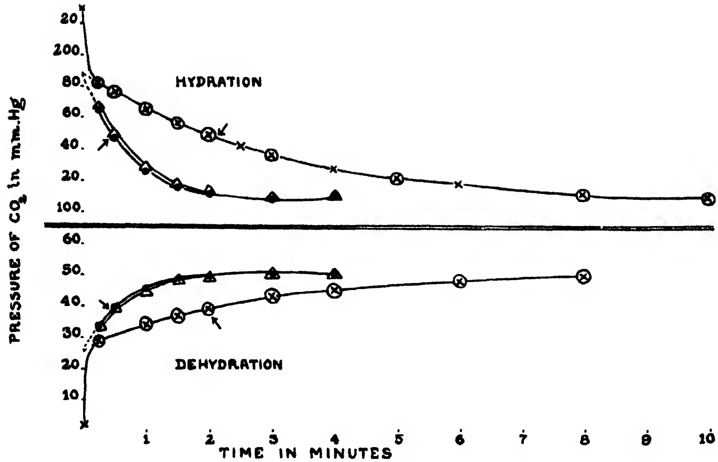

FIG. 2. Reversibility of iodine inhibition of carbonic anhydrase by ascorbic acid. Enzyme Preparation A in 0.2 M phosphate buffer, pH 7.0. Hydration and dehydration were measured in the same solution. Arrows designate times of half hydration and half dehydration. × indicates uncatalyzed reaction; ●, carbonic anhydrase alone; ○, carbonic anhydrase inhibited by iodine (0.5×10^{-3} mole per liter); △, carbonic anhydrase + iodine (0.5×10^{-3} mole per liter) + ascorbic acid (0.01 mole per liter).

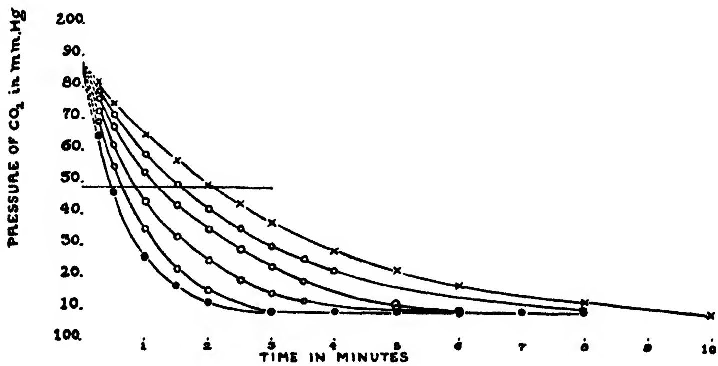

FIG. 3. Partial inhibition of carbonic anhydrase by $KMnO_4$. Enzyme Preparation A in 0.2 M phosphate buffer, pH 7.0. × indicates uncatalyzed reaction; ●, carbonic anhydrase alone; ○, carbonic anhydrase inhibited by $KMnO_4$ in four different concentrations. The horizontal line intersects the curve at the points of half hydration.

tioned that the same result was obtained when the enzyme was inhibited by sulfide or cyanide.

It may be concluded, therefore, that our experiments give no evidence that the hydration function of carbonic anhydrase can be inhibited to a greater or less extent than the dehydration function.

Several reducing agents were tested for their effect upon carbonic anhydrase. Ascorbic acid in a concentration of 0.1 M did not affect the enzyme; 0.05 M cysteine inhibited it slightly, but 0.01 M was without any effect; 0.005 M anthraquinone-β-sulfonate had a very slight inhibiting effect in both the oxidized and reduced state; phenosaphranine, 0.008 M, did not affect the enzyme in either the oxidized or reduced state.[2]

Influence of Hydrogen Ion Concentration

A study of the relation of pH to carbonic anhydrase activity has been made. In the preceding paper, it was shown that the influence of pH on the catalytic effect of hypobromite and sulfite is quite different.

Meldrum and Roughton (1934) observed that the catalytic effect of the enzyme upon the hydration was smaller at pH 10 than at pH 7.6. Booth (1938) studying the effect of a carbonic anhydrase inhibitor in blood presented a curve for the activity of the enzyme in 0.2 M phosphate at pH 6.8 to 8.1. He found that the enzyme activity increased steadily from pH 6.8 to 8.1, being 6-fold larger at 8.1 than at 6.8.

We have investigated the activity of the enzyme in the pH range from 6.1 to 10.1, employing phosphate, pyrophosphate, and carbonate as buffers. Typical results are given in Table II. The concentration of the enzyme Preparation A, expressed in γ per 100 cc., required to double the rate of hydration, has been determined at different pH values. At the same pH, the activity of the enzyme was practically the same in both phosphate and

[2] It may be mentioned that the dyes, brom-thymol blue and phenol red, inhibited the carbonic anhydrase. Both dyes have been used in colorimetric methods for the determination of carbonic anhydrase (Brinkman, 1934; Philpot and Philpot, 1936). In a concentration of 10^{-4} M, brom-thymol blue inhibited the enzyme activity about 50 per cent. Phenol red, in the same concentration, inhibited it to an extent of 10 to 25 per cent.

pyrophosphate. The optimal activity for the total hydration appears to be approximately pH 8.

The hydration of CO_2 proceeds in two ways: $CO_2 + H_2O \rightleftharpoons H_2CO_3$, and $CO_2 + OH^- \rightleftharpoons HCO_3^-$. At present, we cannot decide whether the carbonic anhydrase catalyzes both reactions. Some enzymes have different optimal pH values, depending upon the concentration of substrate. Within the limits of substrate concentration used in our experiments, no differences in optimal pH values were observed for carbonic anhydrase. Our measurements were made with two different substrate concentrations, namely an initial pressure of 100 mm. of CO_2, and 230 mm. of

TABLE II

Effect of pH on Over-All Rate of Hydration

pH	Concentration of enzyme preparation required to double rate of hydration	Buffer
	γ per 100 cc.	*M*
6.10	17.5	0.2, phosphate
6.90	7 0	0.2, "
7 05	7 0	0 1, pyrophosphate
7 54	3 7	0.2, phosphate
7.60	4.0	0.1, pyrophosphate
8 10	2.3	0.1, "
8.55	3.3	0.1, "
9.0	5.0	0.1, "
9.3	6 3	0.1, arsenate
10.1	18.0	0.1, carbonate

CO_2 (all CO_2 in the gas phase), but no difference in the pH curves was found.

Carbon Monoxide

Meldrum and Roughton (1934) reported that carbon monoxide produced considerable inhibition of carbonic anhydrase. This inhibition was stronger in the dark than in the light. We repeated these experiments with different results.

Carbon monoxide was prepared by dehydration of formic acid by sulfuric acid. The gas was washed by an alkaline solution of hydrosulfite and anthraquinone-β-sulfonate, and stored in a reservoir. When drawn from the reservoir, the gas was bubbled

through the hydrosulfite solution, followed by strong sodium hydroxide to remove traces of sulfide from the CO. For experiments in the dark, the reaction chamber was painted black.

The buffer solution with the enzyme was freed from gas by evacuation. The buffer-enzyme mixture was then saturated at a certain CO pressure and kept for different lengths of time. Without shaking, the vessel was then evacuated, the CO replaced by a mixture of CO and CO_2, and the rate of hydration measured as usual.

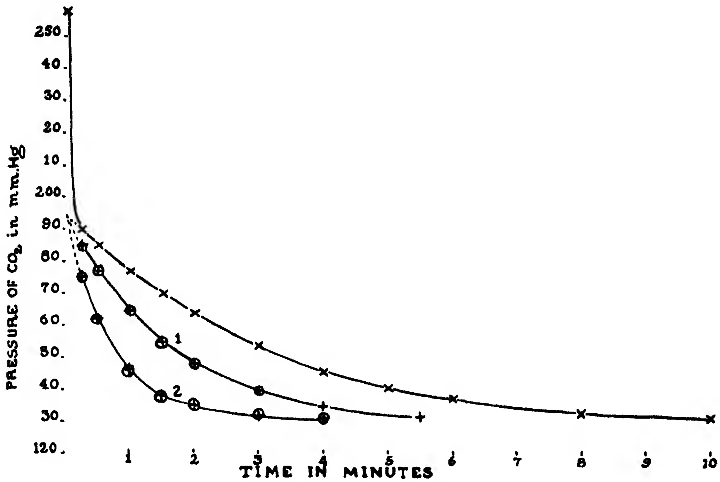

FIG. 4. Activity of carbonic anhydrase in the presence of carbon monoxide. Enzyme in 0.2 M phosphate, pH 6.9. Curves 1 and 2 represent rates with two different amounts of carbonic anhydrase; O, without exposure to CO; +, with exposure to 800 mm. of CO for 5.5 hours; ×, the uncatalyzed reaction.

The CO pressure was varied from 200 to 1200 mm. of Hg and the time of exposure of the enzyme to CO, before the activity was measured, from 10 minutes to 12 hours.

In a series of ten experiments, we did not observe any evidence of inhibition of the carbonic anhydrase. Fig. 4 shows one example of these experiments. We have no explanation for the difference between our results and those of Meldrum and Roughton.

In view of the relation between activity and zinc content,

demonstrated by Keilin and Mann, carbon monoxide might be expected to have no effect on the activity of the enzyme.

Sulfide and Cyanide

Cyanide and sulfide were found by Meldrum and Roughton (1934) and Stadie and O'Brien (1933) to be strong inhibitors of carbonic anhydrase. Our experiments confirm these observations. We found even a higher sensitivity of our enzyme Preparation A to cyanide than Meldrum and Roughton describe. Table III summarizes the experiments on the inhibition by different concentrations of sulfide and cyanide. The experiments were carried out with a concentration of enzyme Preparation A which increased the hydration rate 4-fold. The enzyme activity was inhibited 50 per cent by sulfide, 0.23×10^{-5} M, or cyanide, 0.7×10^{-5} M. Sulfide and cyanide are stronger inhibitors than iodine and permanganate, which required a concentration of 5×10^{-5} mole per liter to inhibit the activity 50 per cent. Sulfide and cyanide did not affect the uncatalyzed reaction.

TABLE III

Inhibition of Carbonic Anhydrase by Cyanide and Sulfide

0.2 M phosphate, pH 7.0. Enzyme concentration = amount necessary to accelerate uninhibited rate of hydration 4-fold.

Concentration of cyanide or sulfide	Inhibition by cyanide	Inhibition by sulfide
moles $\times$ *10^5 per l.*	*per cent*	*per cent*
0.1		18
0.2	22	43
0.5	40	78
1.0	59	96
2.0	70	
4.0	82	
10.0	92	
20.0	100	

SUMMARY

Experiments on the purification of carbonic anhydrase have been carried out and the properties of the enzyme preparation studied.

Hydration of CO_2 and dehydration of H_2CO_3 are influenced by the enzyme equally. The activity of the enzyme may be inhibited by a series of oxidizing agents and the activity restored by certain reducing agents. The effect of the inhibitors was shown to be the same on both the hydration and dehydration activity.

The activity of the enzyme in relation to the hydrogen ion concentration was investigated over a range from pH 6.1 to 10.1. The rate of hydration by the enzyme was found to be optimal at pH 8.1.

Carbon monoxide at pressures up to 1200 mm. of Hg did not inhibit the activity of the enzyme. In confirmation of others, we found sulfide and cyanide to be strong inhibitors of the enzyme.

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STUDIES ON BIOLOGICAL OXIDATIONS

XII. OXIDATIONS AND CARBOHYDRATE SYNTHESIS IN NEPHRITIC KIDNEY SLICES

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The progress made in the field of biological oxidations during the last years has been of such a nature as to warrant application of the findings to studies on the metabolic behavior of tissues in pathological conditions. It is known now that, as a rule, oxidizable substances are first activated by the activating protein[1] (miscalled "dehydrogenase" by some authors); the activated substrate is then oxidized by a series of sluggish and electroactive reversible oxidation-reduction systems which transfer the electrons from the oxidizable substance to molecular oxygen (see Barron's review (1)). In animal tissues the rate of these oxidations is regulated not only by those factors which influence the rate of single reactions, and those factors which determine the orientation of reactions, but also by the influence of hormones, the nature of which is still almost unknown. An alteration by a pathogenic agent of any of these factors may produce a reorientation of reactions, or a diminution or increase in the rate of reactions. The well known studies of Warburg (2), who showed the change in the orientation of reactions (the so called Pasteur reaction) that occur in cancer tissues, are a classic example. Barron (3) found that the tissues of Rous chicken sarcoma and of the infectious myxoma of the rabbit do not oxidize succinic acid, and that the tissues of animals inoculated with filtrable virus diseases oxidize it more slowly than normal tissues. Cross and Holmes (4) on studying

[1] The term "activating protein" has been temporarily retained here until an agreement is reached on the confused nomenclature of the oxidation enzymes.

the effect of diphtheritic toxemia on the metabolism of liver slices of rabbits found the power to form extra carbohydrates in the presence of lactic acid, pyruvic acid, and alanine greatly reduced; they also found a diminution in the rate of oxidation of lactic and pyruvic acids. A decrease in the activity of fumarase was observed by Jacobsohn and da Cruz (5) in the liver of guinea pigs poisoned by $CHCl_3$.

There is reported in this paper a study of the rate of tissue oxidations and carbohydrate synthesis in kidney slices from rats with experimental nephritis, the drug chosen for the production of nephritis being diethylene glycol, the effects of which have been thoroughly studied recently (Kesten, Mulinos, and Pomerantz (6), Cannon (7), Ruprecht and Nelson (8), Linch (9), Geiling and Cannon (10)). It is known that the energy necessary for the maintenance of the normal activities of the kidney, such as excretion, absorption, and modification of metabolites, is drawn from oxidation-reduction processes. In cases of impaired renal function, the oxidative enzyme systems may become altered and this alteration in turn may still further limit the ability of the kidneys to do work. Damage of the enzyme components responsible for oxidation and synthesis must be an important factor in the development of many diseases, and when more is known concerning this problem the mechanisms operating in the production of pathological states will be better understood.

In this paper it is shown that experimental nephritis is accompanied with a diminution in the rate of oxidation of some metabolites and in the rate of carbohydrate synthesis. An attempt was made to discover which of the components of the oxidation enzymes had been altered by the pathological process; it seems that the drug alters the activating proteins.

EXPERIMENTAL

Preparation of Animals—Young mature albino rats were grown on a diet which has been shown by Sherman and Campbell (11) to be completely adequate. The diet consisted of two-thirds whole ground wheat, one-third whole milk powder, and salt to the extent of 2 per cent of the weight of the wheat. Each litter of rats was divided equally into a control group and a group which received diethylene glycol. The drug was given orally in a 1:4

dilution twice daily in doses of 2 cc. per kilo of body weight. The treatment was continued until the animals became anuric, which generally took from 3 to 6 days. The course of the disease was also followed by continual determinations of non-protein nitrogen in the blood.

Preparation of Tissue Samples—Slices of kidney for studies on both tissue respiration and synthesis of carbohydrate were taken from the central part of the kidney, the slice representing a cross-section of both cortex and medulla. Oxygen consumption and CO_2 production were measured with the usual Warburg-Barcroft manometers and Warburg vessels, each experiment being done in duplicate; those results which did not agree within 4 to 5 per cent were discarded. The slices were kept in the bicarbonate and phosphate solutions recommended by Krebs and Henseleit (12).

Determinations—For the determination of carbohydrate synthesis, 80 to 100 mg. of kidney slices were placed in 25 cc. Erlenmeyer flasks containing 4 cc. of Krebs' bicarbonate solution (pH 7.4) and 0.02 M pyruvate. The flasks were stoppered with rubber stoppers and filled with an O_2-CO_2 mixture (95:5) through injection needles which pierced the stoppers. The flasks were shaken continuously in a water bath at 38° for 2 and 3 hour periods, after which there was added 0.4 cc. of 2.5 N HCl. Total carbohydrate determinations were made on the tissue plus the fluid, by the procedure, with a few changes, of Benoy and Elliott (13), who adapted the method of Tsai (14) and West, Scharles, and Peterson (15) to small quantities. To each of the flasks containing the tissue and 4.4 cc. of fluid, 1.1 cc. of 5 N H_2SO_4 were added. Digestion was carried out on the water bath for 3 hours. The tissue slices were broken up with a stirring rod after the first 15 minutes of heating and the stirring rod was washed down with a little 1 N H_2SO_4. The fluid was cooled and transferred quantitatively to a 25 cc. volumetric flask. 1 cc. of a solution of 30 per cent $HgSO_4$ in 10 per cent H_2SO_4 was added to each flask. After diluting to the mark, the contents were poured into a 150 cc. Erlenmeyer flask. 10 minutes later 5 gm. of $BaCO_3$ were added. After the material had stood a second 10 minutes, with occasional shaking, the $BaSO_4$ and excess carbonate were removed by centrifugation. The solution was tested for neutrality with litmus paper and when necessary one drop of 20 per cent NaOH was added. 2 drops of a saturated

solution of Na_2SO_4 were also added at this point. Mercury was removed by adding 0.2 to 0.4 gm. of aluminum powder, shaking in a mechanical shaker for 30 minutes, and filtering. The final glucose determinations were carried out by the micromethod of Somogyi (16).

For the determination of ammonia, the fluid was distilled by steam distillation according to the method of Parnas and Klisiecki

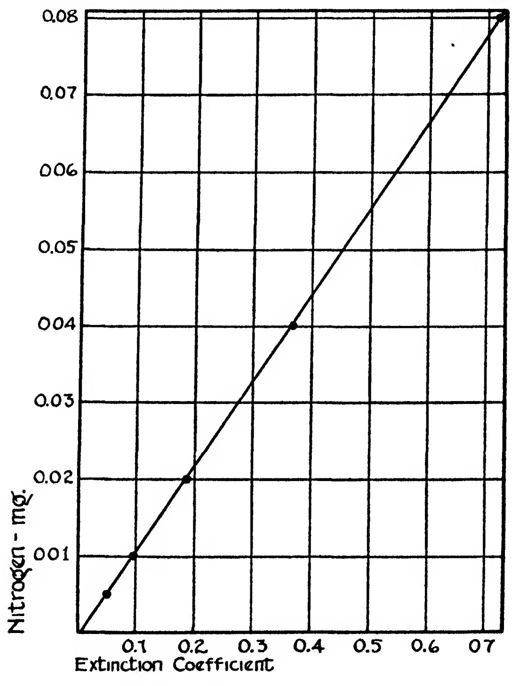

FIG. 1. Standard curve for colorimetric ammonia determination with the Pulfrich photometer. Total volume of nesslerized solution, 20 cc.

(17). After nesslerization, the NH_3 was determined colorimetrically with the Pulfrich photometer, the extinction coefficient being determined with Filter S-43. The use of the photometer allows accurate determinations on much smaller quantities of NH_3 than is possible with an ordinary colorimeter. As can be seen in Fig. 1, the extinction coefficient is strictly proportional to the concentration of nitrogen in the solution. The useful range

of determinations includes samples containing as little as 0.0025 mg. of nitrogen, the quantities determined in this investigation varying from 0.079 to 0.0038 mg. It is necessary to make a standard curve for each batch of Nessler's reagent and also to check the curve after the reagent has stood for 4 or 5 months. For example, a determination of 0.05 mg. of nitrogen with fresh Nessler's solution gave a reading of $E = 1.36$; a year and a half later, the same amount gave a reading of 1.29.

Diphosphothiamine was prepared from thiamine according to the method of Weijlard and Tauber (18), the thiamine having been generously furnished by Merck and Company, Inc., to whom we express our thanks. The lithium salts of lactic and pyruvic acids were prepared at the laboratory. The amino acids were obtained from Hoffmann-La Roche, Inc. Random samples of kidney were taken for microscopic examinations, which were kindly made by Dr. Louis E. Leiter.

Oxidations Produced by Rat Kidney Slices (Normal and Nephritic)—The measurement of the oxygen consumption by kidney slices without added substrate proved of no value for distinguishing the normal kidney from the nephritic. As can be seen in Table I, the average Q_{O_2} values (c.mm. of O_2 uptake per mg. of dry weight per hour) in thirteen animals, normal and nephritic, were the same, 17.6 ± 0.4. Measurements in the presence of glucose gave in the nephritic animals erratic and non-reproducible values, presumably because the metabolism of glucose requires the influence of a number of enzyme systems (of fermentation and oxidation) which may have been altered in different degrees with consequent reorientation of reactions not detectable by simple O_2 consumption measurements. For these reasons it was decided to study the efficiency of individual oxidation enzymes, limiting the study to those oxidations the mechanism of which is reasonably well understood. The substrates chosen were lactate and pyruvate, both important intermediate substances in the metabolism of carbohydrates; succinic acid, because the oxidizing enzyme succinoxidase seems to act as a catalyst for the oxidation of carbohydrates (von Szent-Györgyi (19)); choline, because it seems to affect fat distribution (MacLean, Ridout, and Best (20)); and the amino acids, *dl*-alanine and *l*-aspartic acid. The values quoted in Fig. 2 were obtained by sub-

TABLE I

Oxygen Consumption of Kidney Tissue Slices without Added Substrate

Temperature, 38°; pH, 7.4; in Krebs' phosphate solution.

Control		Nephritic		
Animal No.	O_2 consumption, Q_{O_2}*	Animal No.	O_2 consumption, Q_{O_2}*	Blood non-protein N
				mg. per cent
2	15.5	1	17.6	
3	15.9	4	20.6	
7	15.8	5	17.5	
8	21.6	6	12.9	
19	18.9	9	19.2	
26	17.5	10	15.8	
27	15.1	11	13.8	151
30	19.8	20	16.6	138
33	19.7	21	17.5	
34	13.7	24	19.5	125
39	19.4	28	16.1	125
44	18.3	31	22.6	70
48	17.8	37	16.3	
		45	20.7	155
Average	17.6 ± 0.4		17.6 ± 0.4	

* C.mm. per mg. of dry weight of tissue per hour.

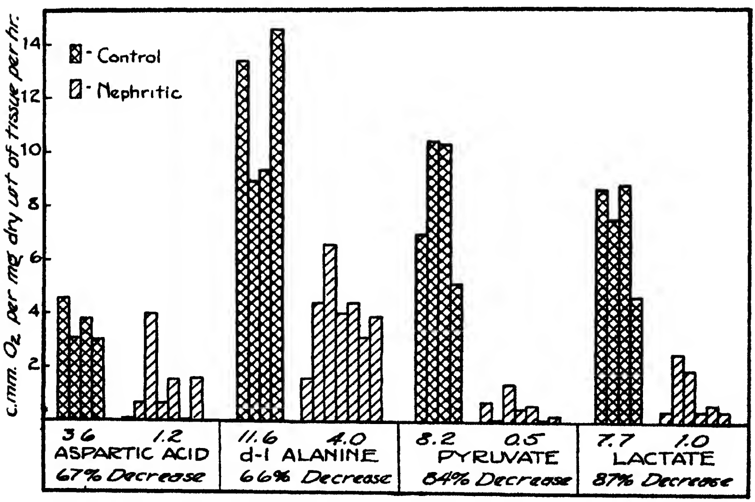

FIG. 2. The oxidation of various substrates by rat kidney tissue. Concentration of substrate, 0.03 M; temperature, 38°; gas phase, O_2; fluid, Krebs' phosphate. The values shown are differences between the oxygen consumption in the presence and in the absence of a given substrate. Each column represents an individual animal.

tracting the oxygen consumption of the tissue alone from the value obtained with the tissue plus substrate. The ability to oxidize pyruvate and lactate was diminished considerably in the nephritic tissue, and in some instances was completely lost. Of particular interest is the loss of ability to oxidize pyruvate, since this substance seems to play an important rôle in the metabolism of animal tissues, not only as an intermediate in the breakdown and synthesis of carbohydrates but also in the synthesis of amino acids.

It is known that the mechanism of oxidation of amino acids is different for the naturally occurring amino acids and for their optical isomers (Krebs (21), Warburg and Christian (22), von Euler *et al.* (23)). As an example of oxidation of naturally occurring amino acids, the oxidation of *l*-aspartic acid was selected; the oxidation of *dl*-alanine was taken as representing the oxidation of the non-natural form, because its oxidative deamination is about 15 times as fast as that of the natural isomer (Krebs (21)). The rate of oxidation of both amino acids was decreased to about the same extent; namely, by 67 per cent in the oxidation of *l*-aspartic acid, and by 66 per cent in that of *dl*-alanine (Fig. 2).

The rate of oxidation of choline was also greatly impaired, the decrease being about 87 per cent (Fig. 3). The oxidation of succinic acid was the least affected of all, the average decrease being about 24 per cent (Fig. 4).

To determine whether diethylene glycol acted *per se* as an ordinary inhibitory agent of cellular oxidations or indirectly through continuous and slow action on the tissues, the effect of *in vitro* addition of the drug on the rate of oxidations of normal kidney slices was studied. The addition of diethylene glycol in concentrations as high as 0.1 M had no effect on the oxidation of lactate, pyruvate, or choline over periods of 1, 2, and 3 hours (Table II). It may be concluded that diethylene glycol is neither a specific inhibitor (such as HCN) nor a general inhibitor (such as narcotics).

Anaerobic Oxidations with Ferricyanide As Oxidizing Agent—Having ascertained that some of the oxidative systems were blocked in the nephritic kidney, we next wished to discover if possible the enzyme component damaged by diethylene glycol. The oxidation of all the substrates studied in this paper requires

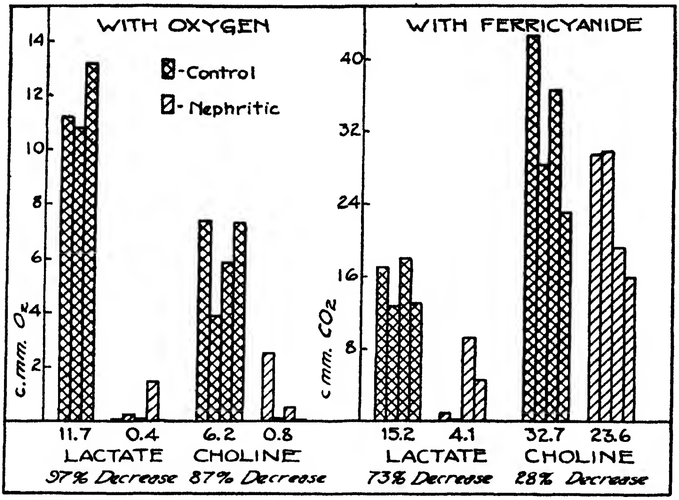

FIG. 3. The oxidation of lactate and choline by rat kidney tissue in the presence of oxygen and also in the presence of ferricyanide as oxidizing agent. Concentration of substrate, 0.03 M; amount of K_3FeCN_6, 0.2 cc. of 9.8 per cent in bicarbonate solution per vessel; temperature, 38°. The values are represented in terms of c.mm. of O_2 consumed or CO_2 evolved per mg. of dry weight of tissue per hour. The consumption of O_2 or the evolution of CO_2 by the tissue without added substrate was subtracted.

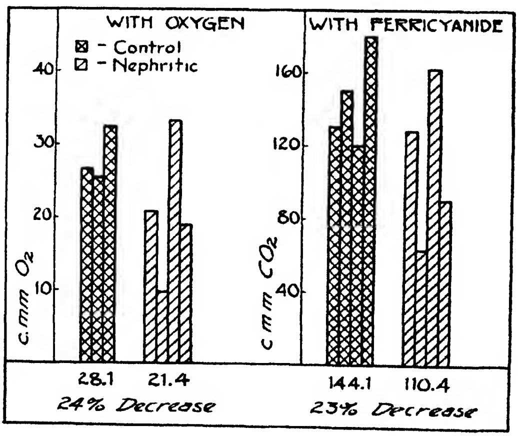

FIG. 4. The oxidation of succinate by rat kidney tissue with oxygen and with ferricyanide. Concentration of substrate, 0.03 M; amount of K_3FeCN_6, 0.2 cc. of 9.8 per cent in bicarbonate solution per vessel; temperature, 38°. The values are represented in terms of c.mm. of O_2 consumed or CO_2 evolved per mg. of dry weight of tissue per hour. The consumption of O_2 or the evolution of CO_2 by the tissue without added substrate was subtracted.

the presence of a specific activating protein and a number of reversible oxidation-reduction systems. In some cases the latter may be replaced by reversible systems of potentials suitable to perform the oxidation; the protein, being specific, cannot be replaced. A convenient method of determining the activity of the protein, introduced by Quastel and Wheatley (24), consists in the use of ferricyanide as the oxidizing agent, the oxidation being followed manometrically in a bicarbonate solution with 95 per cent N_2 and 5 per cent CO_2 as gas phase. Ferricyanide on being reduced liberates CO_2 from bicarbonate, the rate of CO_2 evolution being a relative measure of the rate of oxidation of the substrate. Preliminary experiments were performed to ascertain

TABLE II

Effect of Diethylene Glycol on Oxygen Consumption of Normal Kidney Tissue In Presence of Various Substrates

Diethylene glycol concentration, 0.1 M; temperature, 38°; pH, 7.4. The values are given in c.mm. of O_2 per mg. of dry weight of tissue per hour.

Time	Lactate, 0.03 M		Choline, 0.01 M		Pyruvate, 0.03 M	
	Control	Diethylene glycol	Control	Diethylene glycol	Control	Diethylene glycol
hrs.						
1	30.8	30.6	22.4	23.4	25.6	26.4
2	60.8	62.4	42.2	43.9	54.2	55.3
3			59.0	61.6		
4			72.6	76.9		

that $Na_3Fe(CN)_6$ at the concentration used in these experiments (0.0208 M) had no influence on the rate of oxygen consumption by kidney slices. As reported by Quastel and Wheatley in their experiments with liver slices, it was found that in the kidney also ferricyanide was unable to act as oxidizing agent in the anaerobic oxidation of pyruvate and alanine. In all the experiments the small CO_2 evolution produced by the tissues alone on addition of ferricyanide was subtracted.

The rate of oxidation of lactate was decreased in the nephritic kidney to a large extent whether the oxidation was performed through molecular oxygen or ferricyanide (97 per cent inhibition in the oxidation by atmospheric oxygen, 73 per cent in the oxida-

tion by ferricyanide). On the other hand, there was a difference in the oxidation of choline: while there was a decrease of 87 per cent in the rate of oxidation of choline by atmospheric oxygen, the decrease was only 28 per cent when ferricyanide was used (Fig. 3).

Fig. 4 shows the results obtained with succinate. The rate of its oxidation both by atmospheric oxygen and by ferricyanide was inhibited only slightly (23 per cent) in the nephritic kidney when compared to the rate of oxidation in the normal kidney.

An interpretation of these findings must be made with caution. Lactate is oxidized in animal tissues by the action of the following components: protein, diphosphopyridine nucleotide, alloxazine, and the cytochrome system. Quastel and Wheatley (24) are of the opinion that ferricyanide reacts with reduced diphosphopyridine nucleotide (reduced by oxidation of lactate), since the rate of reduction increased on addition of this substance. Whether the inhibition found in nephritic kidney is due to direct action of diethylene glycol on the protein part of the complete enzyme system, as we believe, or is due to diminished concentration of diphosphopyridine nucleotide cannot be decided until the same experiments are performed after addition of diphosphopyridine nucleotide. An alteration of the activating protein seems to be the explanation of the experiments on the anaerobic oxidation of succinate. Its oxidation requires a protein and the cytochrome system; ferricyanide may replace the latter. The inhibition in the rate of oxidation of succinic acid was the same whether the succinate was oxidized via the cytochrome system (aerobic experiments) or via ferricyanide (anaerobic experiments).

We can offer no explanation for the results with choline. The oxidation of choline seems to require an activating protein and the cytochrome system (Mann, Woodward, and Quastel (25)). Yet, the greater inhibition of the rate of its aerobic oxidation (87 per cent) than of its anaerobic oxidation with ferricyanide (28 per cent) would indicate that some other component besides protein and cytochrome takes part in the oxidation of choline by the kidney, a component which is altered in the nephritic kidney.

Effect of Diphosphothiamine on Oxidation of Pyruvate by Nephritic Kidney—The opinion that diethylene glycol acts by altering the activating protein seems to find confirmation in the experiments

reported in Table III. It has been shown that the rate of oxidation of pyruvate is considerably decreased in nephritic kidney. The pyruvate oxidation system in animal tissues is made up of the activating protein, diphosphothiamine, alloxazine, and the cytochrome system. Addition of 50 micrograms of diphosphothiamine did not increase the extremely slow rate of oxidation of pyruvate.

Synthesis of Carbohydrate by Nephritic Kidney—Benoy and Elliott (13) have shown that kidney slices are able to synthesize carbohydrate from a variety of substances. Good evidence that the product formed is glucose was given by Weil-Malherbe (26). Benoy and Elliott found that the rate of carbohydrate synthesis in the presence of pyruvate is much greater than in the presence of

Table III

Effect of Pyruvate and Pyruvate Plus Diphosphothiamine on Oxygen Uptake of Nephritic Kidney Tissue

Temperature, 38°; pH, 7.4.

Time	Q_{O_2}		
	Without added substrate	With 0.03 M pyruvate	With 0.03 M pyruvate + 50 γ diphosphothiamine
min.			
20	4.7	4.7	5.0
40	10.6	11.1	11.0
60	16.1	16.5	16.3
80	21.8	22.6	22.0

lactate, acetoacetic acid, alanine, and other substances known to give rise to pyruvic acid in animal tissues. For this reason, pyruvate was chosen as the substrate for the synthesis of carbohydrate in the nephritic kidney. Since the oxidation of this substance is greatly impaired in the nephritic kidney, it was reasonable to assume that the same impairment would be found in the rate of synthesis. As can be seen in Table IV, the rate of synthesis of carbohydrate was considerably inhibited in the nephritic kidney. In three normal rats the kidney synthesized in 3 hours from 1.38 to 2.7 times the original amount of carbohydrate; the kidneys of three nephritic animals synthesized in the same time only from 0.11 to 0.49 times the original amount.

Formation of Ammonia from Amino Acids—In a study of

nephritic human kidneys, Krebs (27) found a marked decrease in the ability of the tissue to form ammonia from amino acids. The same findings were obtained by von Leövey (28) in a study of experimental nephritis produced by uranium nitrate in rats. Parallel with the study of the oxygen consumption of kidney slices in the presence of amino acids, a determination was made at the end of the experiment of the formation of ammonia in the fluid in

TABLE IV

Synthesis of Carbohydrate by Rat Kidney Slices in Presence of 0.02 M Pyruvate

The values are given in mg. of glucose per 20 mg. of dry weight. Temperature, 38°; pH, 7.4.

	Animal No.	Placed in H_2SO_4 immediately	Incubated 3 hrs. without pyruvate	Incubated 2 hrs. with pyruvate	Incubated 3 hrs. with pyruvate
Control	38	0.35	0.34	0.65	0.99
	46	0.43	0.45	0.73	1.07
	49	0.44	0.36	0.97	1.33
Nephritic	45	0.37	0.37	0.48	0.55
	43	0.39	0.42	0.50	0.60
	42	0.33	0.45	0.65	0.50

TABLE V

Ammonia Formation by Rat Kidney Slices during Tissue Respiration Experiments

Substrate	NH_3 formation per mg. dry weight of tissue per hr.	
	Control	Nephritic
	c.mm.	*c.mm.*
None	2.1	1.2
l-Aspartic acid, 0.03 M	4.8	2.5
dl-Alanine, 0.03 M	11.2	8.6

which the tissue slices had been suspended. For comparison with tissue respiration experiments, the quantities of ammonia are expressed in terms of c.mm. per mg. per hour (17 mg. of NH_3 = 22,400 c.mm.). Table V shows that the ammonia formation by the nephritic kidney was considerably decreased in the presence of both *l*-aspartic acid and *dl*-alanine.

Pathological Findings—Dr. Louis E. Leiter found in all the

samples of kidney marked hydropic degeneration of the cortical tubules with more or less extended necrosis of the proximal convoluted tubules, frequent dilatation of the loops of Henle, and hyaline casts in the collecting tubes of both cortex and medulla. Nearly all the glomeruli showed distended capsular spaces, sometimes with protein precipitation and swelling of the capillary walls. The microscopic findings of Dr. Leiter were accompanied by the alterations in metabolism reported here. It may be pointed out that Rat 32, the kidney of which was unable to oxidize lactate, presented more extensive microscopic lesions than Rat 37, in which there was some oxidation (80 per cent less than normal). The same differences were observed in the extent of lesions found in a rat with no oxidation of choline and a rat in which there was some oxidation (64 per cent less than normal); also in a rat the kidney of which had almost lost the ability to synthesize carbohydrate and one in which there was left greater power of synthesis.[2] A closer comparison of the degrees of anatomical and functional alterations would have required the use of animals in the different stages of nephritis.

SUMMARY

Kidney slices from rats made nephritic by ingestion of diethylene glycol showed a marked decrease in the ability to oxidize lactate, pyruvate, choline, succinate, and the amino acids, *dl*-alanine and *l*-aspartic acid. In the nephritic kidney the rate of oxidation of lactate and succinate by oxygen and by ferricyanide was inhibited to about the same extent, an indication that the drug acts by altering the activating proteins of the oxidizing enzymes. The rate of synthesis of carbohydrate, as well as of the formation of ammonia from the oxidative deamination of amino acids, was also greatly inhibited. *In vitro* addition of diethylene glycol to normal kidney slices produced no inhibition of the rate of oxidation of those substances mentioned above.

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THE BIOLOGICAL SYNTHESIS OF HIPPURIC ACID IN VITRO*

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The mechanism of the synthesis of hippuric acid *in vivo* is interesting from several points of view. There is the intrinsic interest in a compound found in the urine of many animals, an interest which is heightened by the use of the rate of hippuric acid excretion following the administration of benzoic acid as a clinical test of liver function. This synthesis is interesting also from the point of view of physiological energetics. The formation of hippuric acid from glycine and benzoic acid is attended by a gain in free energy (Table I). In other words the tendency of the reaction, if allowed to proceed spontaneously at 25° or 38°, is not toward synthesis but toward practically complete hydrolysis of hippuric acid (Table III). Yet when benzoic acid is fed, hippuric acid is rapidly synthesized. This synthesis also occurs and can be measured, as shown below, when liver slices are suspended in Ringer's solution containing low concentrations of benzoic acid and glycine. More than half the benzoic acid is converted to hippuric acid. From the thermodynamic data it may be deduced that the enzymatic synthesis of hippuric acid cannot be simply the reverse of its hydrolysis. The hydrolysis can proceed spontaneously; the synthesis must be coupled with an energy-yielding reaction.

Another, possibly more important, point of interest is that the synthesis of hippuric acid resembles in several respects the synthesis of the peptide bond. The group which is formed, the CONH

* An account of most of the work described here was read at the meeting of the American Society of Biological Chemists at Toronto, April, 1939 (*Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **128**, p. cxiv (1939)).

TABLE I

Data for Calculation of Equilibrium Constant of Reaction, Hippuric Acid + Water → Benzoic Acid + Glycine

Substance	Mol. wt.	Heat of formation of solid at 25°, $\Delta H^0_{298\ 1}$	Free energy of formation of solid at 25°, $\Delta F^0_{298\ 1}$	Solubility	Solubility correction, moles per 1000 gm. water undissociated in saturated solution	Free energy of solution, ΔF	Free energy of formation of neutral molecule in aqueous solution, $\Delta F^0_{aq.}$ (neutral molecule)	Ionization constant, pK	Free energy of formation of ion in aqueous solution at 1 M activity, $\Delta F^0_{R^-}$	Hydrolysis involving hippurate and benzoate (ionized hippuric and benzoic acids): Free energy change, ΔF (hydrolysis)	Hydrolysis involving hippurate and benzoate (ionized hippuric and benzoic acids): Equilibrium constant K (hydrolysis)	Hydrolysis involving neutral molecules (undissociated hippuric and benzoic acids): Free energy change, ΔF (hydrolysis)	Hydrolysis involving neutral molecules (undissociated hippuric and benzoic acids): Equilibrium constant K (hydrolysis)
At 25°													
	gm.	*calories*	*calories*	*moles per 1000 gm. water*		*calories*	*calories*		*calories*	*calories*		*calories*	
Benzoic acid	122.1	−92,270	−59,050	0.0280	0.0267	2150	−56,900	4.20	−51,170	−2640	86	−3170	211
Glycine	75.07	−126,240	−88,500	3.33	0.9*	−650	−89,150						
Hippuric acid	179.2	−145,820	−88,550	0.0205	0.0187	2360	−86,190	3.81	−80,990				
Water (liquid)	18.02	−68,320	−56,690										
At 38°													
Benzoic acid			−57,600	0.0426	0.0409	1980	−55,620	4.20	−49,635	−2670	75	−3225	183
Glycine			−86,850	4.264	0.9*	−835	−87,685						
Hippuric acid			−86.050	0.0332	0.0309	2150	−83,900	3.81	−78,470				
Water (liquid)			−56.180										

* Activity coefficient.

group, is the same, and it is in the α position to a carboxyl group. The free energy of formation of the hippuric acid bond is of the same order of magnitude as of the peptide bond (Tables II and III). From the energetic point of view they are uphill tasks of the same order of magnitude.

TABLE II

Data on Free Energy of Hydrolysis of Peptide Bond (Solid State at 25°)

Substance	Free energy of formation of				Free energy of hydrolysis, ΔF (hydrolysis)
	Peptide, ΔF^0	Water, ΔF^0 (liquid)	Amino acid, benzoic acid, or peptide, ΔF^0	Glycine, ΔF^0	
	calories	*calories*	*calories*	*calories*	*calories*
Glycylglycine	−117,750	−56,690	−88,500	−88,500	−2560
Alanylglycine	−116,910	−56,690	−88,740	−88,500	−3640
dl-Leucylglycine	−112,110	−56,690	−83,260	−88,500	−2960
Hippuric acid	−88,550	−56,690	−59,050	−88,500	−2310
Hippurylglycine	−118,080	−56,690	−88,550	−88,500	−2280

TABLE III

Data on Free Energy of Hydrolysis of Peptide Bond in Aqueous Solution at 25°

Substance	Free energy of formation of neutral molecules of				Free energy of hydrolysis, ΔF (hydrolysis)	Equilibrium constant, K (hydrolysis)	Equilibrium degree of hydrolysis of initial concentrations	
	Peptide, ΔF^0 (aqueous)	Water, ΔF^0 (liquid)	Alanine or benzoic acid, ΔF^0 (aqueous)	Glycine, ΔF^0 (aqueous)			0.1 M	1.0 M
	calories	*calories*	*calories*	*calories*	*calories*		*per cent*	*per cent*
Alanylglycine.	−117,560	−56,690	−89,100	−89,150	−4000	866	99.99	99.88
Hippuric acid.	−86,190	−56,690	−56,900	−89,150	−3170	211	99.95	99.55

For these reasons we have begun a study of the mechanism of the biological synthesis of hippuric acid. The use of the whole animal, whether operated on or not, is too cumbersome and slow. The tissue slice technique of Warburg (1) seemed to be better suited to this purpose. A micromethod was necessary for measur-

ing very small quantities of hippuric acid. One was devised by means of which it is possible to measure as little as 2×10^{-3} mg. We were then able to measure the synthesis of hippuric acid effected by tissue slices in the course of a few hours from dilute solutions (0.0025 M) of benzoic acid and glycine.

A survey was made of the distribution of the capacity for synthesizing hippuric acid among the tissues of several animals. The availability of different amino acids for this synthesis and the effect of poisons and damage to cell structure have been studied. These findings, the thermodynamic data, and a description of the technique and analytical method employed are described in the present communication.

Thermodynamic Data

We have described in previous communications both the general principles and the details of calculating equilibrium constants from thermal data (2–4). We shall therefore present here only the necessary data and the final results of the calculations. The values for the free energy of formation at 38° were calculated from the values at 25° by means of the van't Hoff equation, on the assumption that ΔH is constant over the temperature interval from 25–38°.

The thermodynamic data in Tables I to III are based on measurements of heat capacities and heats of combustion made in this laboratory by Dr. H. M. Huffman. The details of this work have not yet been published, though one compilation of part of these data has been published (5). The thermal data in Table I differ from those given in this previous compilation. The differences are formal only, and arise from a change in the accepted value for the heat of combustion of benzoic acid. The values for the heat and free energy of formation of liquid water at 25° are those of Rossini (6).

The solubility of benzoic acid was obtained from Seidell (7). The value at 25° agrees closely with that determined later by Kolthoff and Bosch (8). The solubility of glycine at 25° and 38° as well as the activity coefficient in the saturated solution are taken from Schmidt (5). We have assumed that the activity coefficient at 38° is the same as that determined experimentally at 25°. The possible error involved is negligible for our present

purposes. We have ourselves determined the solubility of hippuric acid at 25° and 38°. Our value at 25° is identical with that given by Kendall (9).

A number of determinations have been reported of the thermodynamic ionization constant of benzoic acid. The most reliable and concordant value is that obtained at 25° by Brockman and Kilpatrick (10), and by Saxton and Meier (11). The value for the thermodynamic ionization constant of hippuric acid we have used is that given by Josephson (12).

There are no reliable values for the ionization constants of these two acids at 38°. The values we have used are the experimental values at 25°. This seemed preferable to guessing the change in the ionization constants with temperature. It is exceedingly improbable that the difference between the ionization constants of the two acids is, for our purpose, significantly different at 38° from what it is at 25°. It is the difference in the ionization constants which contributes to the final over-all change in free energy. These ionization constants were used to compute the ionization of the two acids in their saturated solutions.

The equilibrium constant for the hydrolysis of hippuric acid in water has been measured experimentally at 184° and 194° by Ingersoll and Burrows (13). The temperature difference is too small, and uncertainties regarding degrees of ionization and activity coefficients at these temperatures too great, for a reliable extrapolation of the equilibrium constant from the higher temperatures to 25°. Without any corrections, the extrapolated value at 25° for the reaction between neutral molecules is 546, which corresponds to a value of ΔF of −3735 calories. The more reliable value calculated from the thermal data is −3170 calories. The agreement is surprisingly close.

The findings on the enzymatic hydrolysis of hippuric acid are in complete accord, as far as they go, with these thermodynamic data. Thus Mutch (14) prepared an extract of hog kidney which hydrolyzed 0.08 N hippuric acid 97 per cent in 255 hours at 37°. The hydrolysis was prevented from being complete by the inhibition of the enzyme, demonstrated by the same author, by the accumulating benzoic acid. A saturated solution of sodium benzoate and glycine set away with the enzyme for 1 month at 37° yielded a small amount of impure, unidentified material, which

at the most, had it been hippuric acid, would have amounted to 0.6 per cent conversion of the benzoic acid to hippuric acid. Kimura (15) obtained complete hydrolysis of hippuric acid with a glycerol extract of hog kidney. Takahashi (16) found nearly complete hydrolysis when sodium hippurate was incubated with an aqueous suspension of minced chicken kidney. He recovered in one experiment 81 per cent of the theoretical benzoic acid and 90 per cent of the amino nitrogen freed.

Technique

Most of the animals used in these experiments were Wistar Institute adult white rats. When other animals were used, the technique was the same. The animals were all in a normal state of nutrition, and were killed by stunning. The organs were sliced free-hand with a straight edge razor. A well sharpened straight edge razor is a better tool for slicing than any safety razor blade we have tried.

The slices were rinsed in Ringer's solution prepared according to the formula of Krebs and Henseleit (17), containing 0.2 per cent glucose, and equilibrated at 38° with a gas mixture consisting of 95 per cent oxygen and 5 per cent carbon dioxide. Each slice was rinsed immediately after being cut and then transferred to the reaction vessel.

Since we were not interested in the respiration of the slices, we have been using 25 ml. bottles as reaction vessels instead of Warburg respirometers. Into these are fitted 2-hole rubber stoppers provided with inlet and outlet tubes for the passage of the gas mixture. The inlet tube reaches half-way into the bottle. It is connected to a glass manifold, which in turn is connected to a cylinder containing the gas mixture. The flow of gas through each reaction vessel is regulated by means of a screw-clamp. One end of the outlet tube in the reaction bottle is flush with the bottom of the stopper; the other end passes through a ring seal into a glass bulb which contains water and has a hole near the top. The bubbling of the gas through the water in this bulb enables one to estimate the rate at which the gas mixture is passing through the reaction vessel.

Twenty-four such reaction vessels are clamped on three strips of wood in such a manner that the lower half of each vessel is

immersed in the water bath. These strips are attached at each end to an upright strip which is mounted on the usual rocking device for Warburg respirometers.

When all the reaction vessels are in place and connected, the gas mixture is blown through vigorously for 10 minutes, after which, for the remainder of the experiment, the gas is slowed down so that about one bubble escapes in the traps about every 2 seconds. Throughout the period of gassing and afterwards the vessels are rocked at a rate of about 90 cycles per minute. The temperature of the water bath was 38°.

This arrangement is much cheaper and more convenient than the usual all-glass respirometer assembly; it is more rugged, and serves quite as well.

Analytical Procedure

The method employed for the analysis of hippuric acid is in principle the classical method of extraction, hydrolysis, and formol titration of the liberated glycine. The extraction apparatus is shown in Fig. 1. Bulb *C* in which the extraction occurs is filled with solid glass beads 3 mm. in diameter to increase the ether-water interface. The receiver *D* is a test-tube attached by a ground glass joint to the extractor. During the extraction *D* rests in a water bath at 75°. Fitting into *A* is a Hopkins condenser. *D* and *A* have the same diameter, so that the condenser will fit as snugly into it as it does into *A*. *D* is about 4 cm. longer than *A*, so that the condenser will not reach too far down when it is used as a reflux condenser in the hydrolysis of the hippuric acid which has been extracted. The tube *B* must be tall enough to carry a column of ether which can overcome the hydrostatic pressure of the water in the lower part of *C* and the layer of ether above it.

The analytical procedure is as follows: Proteins are removed by boiling or by precipitation with trichloroacetic acid (final concentration 2 per cent). 2 ml. of protein-free solution acidified to pH 1.5 to 2.0 with H_2SO_4 are pipetted into *B*. Ether is pipetted into *B* until enough has overflowed from *C* to a depth of about 1 inch at the bottom of *D*. The condenser *A* with water running through it is then inserted, and the temperature of the bath in which *D* is held is raised to 75°. The other parts of the

extractor are at room temperature. The extraction proceeds for 4 hours. The extractor is then withdrawn from the receiver *D*, from which the ether is then evaporated off. *D* is then removed from the bath to an adjacent hot-plate and its position adjusted so that the bottom is about 1 mm. above the hot-plate. 1 ml. of concentrated HCl is pipetted into *D*, the condenser inserted, and the heater adjusted so that the HCl boils gently. The hydrolysis is carried on for 2 hours. At the end of this time *D* is again

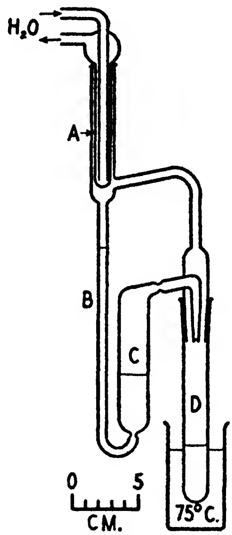

Fig. 1. Apparatus for extraction and subsequent hydrolysis of hippuric acid. *A*, Hopkins condenser; *B*, tube; *C*, extracting chamber filled with solid glass beads 3 mm. in diameter; *D*, receiver.

immersed in a boiling water bath, and the HCl and volatile acids driven off with the aid of a moderately strong current of air, until a piece of moistened Congo red paper held over the top no longer turns blue. The residue in *D* is now dissolved in 2 ml. of water and shaken for a few minutes with permutit to remove any ammonia. After centrifuging, a formol titration is carried out on 0.25 ml. aliquots by the method described in a previous publication (18). The size of the aliquot may be adjusted according to the amount of hippuric acid present.

It is possible by this procedure to measure as little as 2 to 5 γ of hippuric acid in an aliquot titrated. Tested on known amounts of hippuric acid, the recovery was uniformly about 85 per cent. Since macromethods yield only 90 to 95 per cent, the above submicromethod may be considered as tolerably satisfactory. It is sufficiently accurate for our present purposes.

Results

Table IV is a summary of our findings on the synthesis of hippuric acid by slices of liver and of kidney of the dog, guinea pig,

TABLE IV

Formation of Hippuric Acid from 0.0025 M Benzoic Acid and 0.01 M Glycine by Slices of Liver and Kidney of Different Animals, at 38° in 6 Hours

Tissue dry weight, 30 to 50 mg. in 4 ml. of solution.

	Liver				Kidney			
			Q(hippuric acid)	Per cent of initial benzoic acid converted			Q(hippuric acid)	Per cent of initial benzoic acid converted
	mg.	*m.eq.* $\times 10^3$			*mg.*	*m.eq.* $\times 10^3$		
Dog	0	0	0	0	1.15	6.5	0.94	65
Guinea pig..	1.34	7.6	0.59	76	0.93	5.3	0.41	53
Rabbit..	1.34	7.6	0.59	76	1.04	5.9	0.47	59
Rat	1.04	5.9	0.35	59	0.33	1.8	0.12	18

rabbit, and rat. They are in complete accord with the findings in intact animals.

Von Bunge and Schmiedeberg (19) were the first to show that in the dog synthesis of hippuric acid occurs in the kidney, and further that in this animal the kidney is the only site of this synthesis. Both conclusions were confirmed by Snapper *et al.* (20) and by Quick (21).

In the rabbit and probably most other animals synthesis of hippuric acid is not restricted to the kidney. The question was left unsettled in experiments on the whole animal whether or not any hippuric acid synthesis can occur in rabbit kidney (22, 23). Hippuric acid synthesis in rabbit liver was proved by Friedmann and Tachau (24) by isolation of hippuric acid after perfu-

sion of the liver with benzoate and glycine. Snapper *et al.* found in perfusion experiments that the kidney of man, pig, and sheep can synthesize hippuric acid. In view of this finding the clinical interpretation of the rate of excretion of hippuric acid following the ingestion of sodium benzoate as a measure of liver function calls for some caution. That it can be used at all as a test of liver function indicates that hippuric acid synthesis probably occurs in human liver as well as kidney. The ability of the liver of the sheep and pig to carry out this synthesis remains to be investigated.

The concordance between the findings in the whole animal and with tissue slices attests to the reliability of the information obtained with the latter technique. This technique is far superior to experiments on the whole animal or perfusion experiments with respect to convenience, the possibility of setting up blank controls, and of testing a variety of experimental conditions on the identical specimen of the organ. The efficiency of the tissue in the slices is of the same order as in perfusion experiments. Thus the Q(hippuric acid) by dog kidney slices in Table IV is 0.94; in perfusion experiments it was 0.77 and 0.25 (20, 25).

The synthesis of hippuric acid in rat liver slices is a relatively slow reaction compared with the synthesis of urea (17) or of amino acids (26) (Table V). The rate is, on the other hand, faster than the conversion of glycocyamine to creatine.[1]

The slowness with which hippuric acid is synthesized in rat liver slices is compensated for to some extent by the continuation of the reaction with small diminution in rate for at least 6 hours. This is shown in Table VI. The progressive falling off in rate is probably the result of lessening vitality of the slices and diminishing concentration of benzoic acid.

There appears to be an upper limit to the amount of hippuric acid synthesized under our experimental conditions. When more and more tissue is added to the reaction vessel, a point is reached at about 35 mg. of dry weight of tissue at which more tissue does not effect the conversion of more benzoic acid (Table VII).

The rate of synthesis expressed as a fraction of the original amount of benzoic acid is nearly the same with initial concentra-

[1] Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, in press.

TABLE V

Speed of Formation at 38° of Hippuric Acid from 0.005 M Glycine and 0.0025 M Benzoic Acid Compared with Formation of Other Substances by Rat Liver Slices

$$Q = \frac{\text{amount formed computed as if gas in c.mm. (S.T.P.)}}{\text{dry weight of tissue in mg.} \times \text{hrs.}}$$

	Q
Glycine and benzoic acid → hippuric acid ..	0.3 - 1.0
Glycocyamine → creatine*	0.05
$CO_2 + 2NH_4$ → urea.† Without ornithine	1 - 4
With ornithine.	7 -15
NH_4 + pyruvic acid → amino acid‡ ..	1 - 2

* Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, in press.
† Krebs and Henseleit (17).
‡ Neber (26).

TABLE VI

Variation with Time of Rate of Synthesis of Hippuric Acid by Rat Liver Slices from 0.0025 M Benzoic Acid and 0.01 M Glycine at 38°

Tissue dry weight, 24 to 38 mg. in 4 ml. of solution.

Time	Per cent of initial benzoic acid combined	Q(hippuric acid)
hrs.		
2	32	1.03
4	47	0.88
6	67	0.80
8	69	0.60

TABLE VII

Dependence of Rate of Synthesis of Hippuric Acid on Amount of Liver Used in 6 Hours at 38° from 0.0025 M Benzoic Acid and 0.01 M Glycine

Dry weight of liver slices in 4 ml. solution	Per cent of initial benzoic acid converted	Q(hippuric acid)
mg.		
9	29	1.3
14	29	0.77
35	57	0.61
65	63	0.37
98	55	0.21

tions of benzoic acid ranging from 0.005 M to 0.00125 M (Table VIII). At 0.01 M the fraction converted is less than at the lower concentrations, but 0.01 M benzoic acid can hardly be considered toxic because the highest value of Q(hippuric acid) was obtained with this concentration of benzoic acid. The absolute amounts

TABLE VIII

Rate of Hippuric Acid Synthesis from Different Initial Concentrations of Benzoic Acid and 0.01 M Glycine in 6 Hours at 38°

Tissue dry weight, 54 to 80 mg. in 4 ml. of solution.

Initial concentration of benzoic acid	Per cent of initial amount converted	Q(hippuric acid)
moles per l.		
0.01	20	0.55
0.005	53	0.49
0.0025	61	0.31
0.0017	59	0.16
0.00125	52	0.12

TABLE IX

Rate of Hippuric Acid Synthesis from Different Initial Concentrations of Glycine and 0.0025 M Benzoic Acid in 6 Hours at 38°

Tissue dry weight, 125 to 150 mg. in 4 ml. of solution.

Initial concentration of glycine	Per cent of initial benzoic acid converted	Q(hippuric acid)
moles per l.		
0.02	73	0.16
0.01	67	0.16
0.005	52	0.12
0.0025	41	0.09
0.00125	32	0.07

of hippuric acid synthesized grow less with decreasing initial concentrations of benzoic acid.

A somewhat similar relation exists between the rate of hippuric acid synthesis and the initial concentration of glycine (Table IX). The low Q(hippuric acid) values are the result of having employed unnecessarily large amounts of tissue (see Table VII).

We have tested a number of amino acids for their ability to combine with benzoic acid in rat liver slices (Table X). The most

rapid combination occurred with glycine. A large scale experiment was carried out with benzoic acid and glycine and the hippuric acid formed was isolated. The yield on isolation was 75 per cent of that indicated by titration. It was proved to be hippuric acid by melting point determination, 187° (corrected), mixed melting point, and by electrometric titration. A 0.2805 ml. aliquot of a solution of 44.6 mg. in 25 ml. of water was neutralized by 74.1 ml.$^{-3}$ of 0.0375 N NaOH; theoretical titer, 74.5 ml.$^{-3}$.

TABLE X

Synthesis of Hippuric Acid by Rat Liver Slices from Benzoic Acid and Different Amino Acids in 6 Hours at 38° Compared with Amount Obtained with Glycine in Simultaneous Experiments from Same Lobe of Liver

Benzoic acid initially 0.0025 M; amino acid 0.01 M.

Amino acid	Per cent of amount synthesized with glycine	Amino acid	Per cent of amount synthesized with glycine
d-Alanine	0	*d*-Lysine	0
d-Arginine	0	*dl*-Methionine	0
l-Aspartic acid	0	*d*-Ornithine	0
l-Asparagine	0	*dl*-Phenylalanine	0
d-Citrulline	0	*l*-Proline	0
l-Cysteine	0	*l*-Hydroxyproline	40 (?)
l-Cystine	0	*dl*-Serine	50 (?)
d-Glutamic acid	0	*d*-Threonine	0
" " + glycine	80	*l*-Tryptophane	0
Glycine	100	*l*-Tyrosine	0
l-Histidine	0	*d*-Valine	0
l-Leucine	0		

Of all the other amino acids only hydroxyproline and serine gave a positive titer. In the case of serine we are certain that the product formed is not hippuric acid. All that can be said at present is that under our experimental conditions a product is formed by the liver slices which is extracted by the ether and gives in our analytical procedure a significantly positive formol titration.

We obtained some evidence that glutamic acid inhibited somewhat the formation of hippuric acid both in the blank, *i.e.* benzoic acid without glycine added to liver slices, and also with glycine.

From the failure of all the amino acids we have tried (except glycine) to participate in the formation of hippuric acid we may conclude that, at least under the conditions of these experiments, none of the other amino acids is converted to glycine in the liver. The glycine necessary for the hippuric acid which is formed when benzoic acid was added without glycine (it was about 5 per cent of the amount formed with glycine) can easily be accounted for by the autolysis of the liver slices in the course of the experiment (27). We may therefore have to look to other organs, or to materials other than amino acids for the synthesis of glycine in the animal body. The possibility remains, of course, that glycine is formed in the liver from amino acids under conditions other than those which existed in the slices in these experiments.

We have investigated the possibility of the synthesis of hippuric acid from benzoic acid and glycine in minced tissues and tissue extracts. So far all the results have been negative. We have tried minced liver and aqueous and glycerol extracts of liver (rat), and glycerol extracts of the kidney (rat and horse).

Here, we have been unable to confirm the observations of Waelsch and Busztin (28). These authors reported that glycerol extracts of horse kidney effected a very rapid synthesis of benzoic acid and glycine to hippuric acid. In 6 hours at 37° more than 60 per cent of the benzoic acid was converted. The hippuric acid formed was isolated and identified. Depending on the conditions, there was also a varying amount of benzamide formed. These are the first observations to be reported of the synthesis of hippuric acid from benzoic acid and glycine in a tissue extract. They would indicate that in a glycerol extract of kidney the whole system, consisting of the enzyme acting upon the benzoic acid and glycine, the energy-donating enzyme and substrate, and the mechanism by means of which these two are coupled, is preserved intact. Furthermore the synthesis, according to Waelsch and Busztin, proceeds under what are practically anaerobic conditions. Synthesis through "mass action," *i.e.* through reduction of the concentration of water by the glycerol, which is present in a concentration of 30 per cent, is excluded, because the vapor pressure of water in 30 per cent glycerol, and therefore the active concentration of free water, is about 90 per cent of that in pure water (29).

Duplicating the conditions of their experiment as exactly as possible, we could find no trace of hippuric acid synthesis. The benzoic acid we added remained uncombined, and was recovered quantitatively. We were also unable to confirm the extraction procedure of Waelsch and Busztin. In their hands shaking six times with ether (amount not specified) was sufficient to remove the benzoic and hippuric acids from the 30 per cent glycerol-water-protein mixture containing a final concentration of 2 N H_2SO_4. With this procedure we could recover added benzoic acid only incompletely and very little of added hippuric acid. We found it necessary to employ an exhaustive continuous extraction for 2 hours in order to extract these two acids completely from the glycerol-water mixture. We are at a loss to account for our inability to confirm the striking observations reported by Waelsch and Busztin.

It is pertinent in this connection that a glycerol extract of kidney—of the pig in most cases—has been used as a source of histozyme; *i.e.*, of the enzyme hydrolyzing hippuric acid (15, 30-32). Thus Kimura (15) reported that glycerol extracts of pig kidney and liver completely hydrolyzed hippuric acid in 68 to 140 hours at 37°. In 24 hours the most potent glycerol extracts effected 50 to 60 per cent hydrolysis.

We have found that synthesis of hippuric acid by liver slices is completely inhibited by 0.001 M KCN and by treatment with toluene. We may conclude therefore that not only intact cell structure but also cell respiration is essential for the synthesis. This is to be expected from the thermodynamic data.

We have found further that liver slices treated with cyanide did not hydrolyze hippuric acid when it was added to the Ringer's solution. If the synthesis of hippuric acid were simply the reverse of hydrolysis brought about by a shift of the equilibrium through coupling with an energy-donating reaction, it may be expected that when this coupling is broken by a respiratory poison hydrolysis of any hippuric acid present would ensue. This would have been analogous to the hydrolysis of protein in autolysis. The finding that there is no hydrolysis in liver slices poisoned with cyanide indicates that the synthesis of hippuric acid under the conditions we have employed, and probably also under those *in vivo*, is not simply the reverse of hydrolysis.

Quick (33) made the interesting observations that when glycuronic acid monobenzoate was injected into the dog some hippuric acid appeared in the urine; and, conversely, after the injection of hippuric acid glycuronic acid monobenzoate appeared in the urine. Hippuric acid can therefore be both hydrolyzed and synthesized in the dog. This does not indicate that the conjugation of benzoic acid with glycine in the dog or elsewhere is a "reversible" reaction which obeys the law of mass action, in the same sense for example as an ionic reaction, in which the reaction can be moved to the "right" or "left" simply by changing the concentrations on the "left" or "right." The equilibrium is so far over on the side of hydrolysis of hippuric acid that no conceivable increase in benzoic acid and glycine could *per se* lead to a significant amount of hippuric acid being synthesized. This negative result was observed by Mutch (14). The hydrolysis and synthesis of hippuric acid in the dog is an example of a reaction which is physiologically but not physicochemically "reversible," as reversibility is ordinarily understood. The simplest explanation would appear to be that hippuric acid is synthesized in the kidney of the dog and hydrolyzed in other organs.

SUMMARY

1. Thermodynamic data are presented pertaining to the free energy of formation of hippuric acid and of peptides.

2. From these data the equilibrium constants at 25° and 38° for the reaction hippuric acid $\rightleftarrows$ benzoic acid and glycine are calculated.

3. The magnitude of the equilibrium constants so obtained indicates that the synthesis of hippuric acid *in vivo* must be a coupled reaction of which one of the components is an energy-donating reaction. The synthesis cannot be simply the reverse of hydrolysis. This deduction is in accord with the experimental findings.

4. A micromethod is described for determining 3 γ of hippuric acid in 1 to 4 ml. of solution.

5. A technique is described by means of which it is possible to observe the synthesis of hippuric acid effected by slices of liver and kidney.

6. By this technique it was found that in the guinea pig, rab-

bit, and rat the conjugation of benzoic acid with glycine can occur in both the kidney and liver. In the dog it occurs in the kidney, but not in the liver.

7. In rat liver the reaction is relatively slow, one-tenth to one-hundredth that of the synthesis of urea from ammonia.

8. The influence of such factors as time, concentration of benzoic acid and of glycine, and of amount of tissue on the rate of synthesis with rat liver slices is described.

9. Of twenty-three amino acids and amides only glycine led to the formation of hippuric acid by rat liver slices.

10. This synthesis does not occur when the cell structure is destroyed by maceration or when the intact tissue is poisoned with toluene or cyanide. Physically intact rat liver slices poisoned with cyanide do not hydrolyze hippuric acid. These findings are shown to be in accord with deductions from the thermodynamic data.

11. An interpretation of the observed hydrolysis and synthesis of hippuric acid in the whole animal (dog) is presented in the light of the above thermodynamic and experimental data.

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THE DETERMINATION OF ARGININE BY MEANS OF FLAVIANIC ACID*

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Recent interest in the stoichiometric relationships between the quantities of amino acids that can be isolated from a protein and of these in turn to the molecular weight of the protein, as estimated by physicochemical methods, has served to emphasize the necessity for the development of more reliable methods to determine amino acids. The very multiplicity of the methods that have been described is evidence, not only of the importance that is attached to the information they furnish, but also of the dissatisfaction that has been felt with many of them.

Much attention has been given in this laboratory to the problem of determining the basic amino acids with the highest attainable degree of precision, but it is recognized that the large quantity of protein needed, if this is to be achieved by the methods that have been employed, places a serious restriction on general application of the results. It has therefore seemed essential to develop simpler and if possible less extravagant methods for the determination of these substances.

In the present paper a method to determine arginine is described by means of which highly reproducible and apparently accurate results may be secured with a few gm. of protein. The procedure is a fundamental modification of the flavianic acid method of Kossel and Gross (1). Although reference has been made to the use of their method in several laboratories, no thorough study of the conditions under which flavianic acid may best be used as a reagent for arginine seems to have been attempted.

* A preliminary report on this method was presented at the meeting of the American Chemical Society at Boston, September, 1939.

Outline of Method

A sample of hydrolysate that represents from 3 to 5 gm. of protein, freed from as much hydrochloric acid as possible by repeated evaporation and decolorized with norit, is treated in the cold with 4 to 5 moles of flavianic acid per mole of arginine, calculated from the best available information on the yield of arginine from the protein. The arginine, which separates slowly but completely as the *diflavianate* under these conditions, is removed, dissolved in ammonia, and the base is reprecipitated from hot solution as the *monoflavianate* by acidification. The weight of this salt furnishes a measure of the arginine content of the hydrolysate.

The results obtained are compared in Table I with analyses by the silver precipitation method, when possible the *same* preparations as those previously analyzed in this laboratory being employed. In all but two cases higher values have been secured by the present method. Data obtained by other workers with entirely different methods are also given for comparison. When necessary these other data have been recalculated to per cent of arginine by the use of the nitrogen content of the protein, also given in Table I.

Preparations Analyzed

A number of the protein preparations available were highly purified specimens and the analytical values secured from these presumably have significance for the discussion of molecular composition. In addition, a few preparations of proteins, now known to be inhomogeneous, and three tissues are included, either because careful analyses by other methods were available, or because of their general interest. These results serve chiefly for comparison. Unless otherwise indicated, the preparations were made in this laboratory.

Edestin—Made in 1928 by the same method as that employed for the material previously analyzed by the silver precipitation method (3). A very fine preparation made many years ago by Osborne was also analyzed.

Tobacco Seed Globulin—Made in 1931 by dilution and cooling of a warm N sodium chloride extract of fat-free tobacco seed meal and purified by a second separation in the same way (6).

TABLE I

Arginine of Proteins As Determined by Diflavianate Method and by Other Methods

Figures in parentheses refer to the bibliography. Arginine data, except those in the third column, are expressed as per cent of arginine in the protein.

Protein	Diflavianate method				N content of protein	Other methods			
	Arginine	Arginine N of protein N	No. of determinations	S content of monoflavianate. Theory 6.56		Silver precipitation method	Arginase method	Van Slyke method	Flavianic acid method
	per cent	*per cent*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Edestin	16.76 ± 0.07	28.9	8	6.60	18.69 (2)	15.81 (3)	15.5 (4)	15.71 (5)	14.5 (1)
Tobacco seed globulin	16.09 ± 0.09	27.8	4	6.59	18.64 (6)	13.6 (6)			
Cottonseed globulin	14.92 ± 0.06	25.8	4	6.60	18.64 (2)	13.5 (7)			
Arachin (peanut)	13.94 ± 0.07	24.5	4	6.66	18.29 (8)	12.54 (9)			17.54 (1)
Amandin (almond)	13.31 ± 0.05	22.1	4	6.59	19.32 (10)	11.85 (11)			
Gliadin (wheat)	2.57 ± 0.02	4.7	4	6.54	17.66 (2)	2.92 (12)	2.64 (4) 2.92 (13)	3.13 (5) 2.57 (14)	
Zein (maize)	1.60 ± 0.03	3.2	8	6.52	16.13 (2)	1.60 (15)			
Casein	3.72 ± 0.04	7.7	11	6.56	15.52 (16)	3.85 (16)	3.85 (4) 3.74 (17) 3.93 (13)	3.70 (5)	4.40 (1)
Hemoglobin (horse)	3.59 ± 0.07	6.92	12	6.56	16.69 (18)	3.32 (18)	3.92 (4)*	4.07 (20)	4.38 (19)
γ-Pseudoglobulin (horse)	2.66 ± 0.04	7.75	4		15.6				
Fibrin (cattle)	7.70 ± 0.09	14.8	4	6.68	16.77	5.52 (21)	7.45 (4)	7.24 (5)	7.70 (22)
Gelatin	8.68 ± 0.05	15.3	4	6.54	18.27	7.62 (23)	8.90 (4)	8.34 (5)	9.34 (1)
Egg albumin	5.66	11.7	1		15.51 (24)	5.56 (25)	4.99 (4)		5.96 (19)
Hair (human)	9.37 ± 0.05	17.8	4	6.66	16.93	7.96 (26)		8.73 (27)	
Wool (sheep)	10.4 ± 0.1	20.2	4	6.55	16.57 (28)	7.8 (28)	10.3 (13)	10.2 (29)	
Silk fibroin	0.76 ± 0.02	1.3	4	6.59	18.99 (30)	0.74 (30)			1.52 (19)

* This figure is calculated from Hunter and Dauphinee's value of 7.72 per cent of arginine N in globin N on the assumption that horse hemoglobin contains 2.1 per cent of hematin (19).

Cottonseed Globulin—Made in 1927 by a similar method.

Arachin—Made from Spanish peanut meal by Johns and Jones, of the Bureau of Chemistry and Soils, in 1917 (8).

Amandin—Made from fat-free almond meal in 1915 by the customary method (10).

Gliadin—Made in 1939 by a procedure in which precipitation with acetone from a concentrated ethyl alcohol solution was employed as an additional purification step. The initial material was obtained as described by Nolan and Vickery (31).

Zein—Made in 1939 by the method described by Nolan and Vickery (31) and further purified by treatment with acetone.

Casein—Made in 1933 by a method based on the Hammarsten procedure. This sample had been previously analyzed by the silver precipitation method by Vickery and White (16).

Hemoglobin (Horse)—Made in 1928 in Professor Cohn's laboratory at the Harvard Medical School by Dr. Arda A. Green; twice crystallized and subsequently coagulated by heat; previously analyzed for bases in this laboratory (18). A second preparation from the same source, made recently, was also analyzed.

γ-Pseudoglobulin (Horse)—Made in Professor Cohn's laboratory in 1939 by Dr. Green (32).

Fibrin (Cattle)—Made from fresh cattle blood in 1914; purified by exhaustive extraction with solvents.

Gelatin—Made in 1914 from high grade commercial gelatin by precipitation at faintly acid reaction with alcohol; subsequently redissolved in water, neutralized, and reprecipitated with alcohol.

Egg Albumin—Made in Professor Cohn's laboratory in 1938; crystallized five times from ammonium sulfate, coagulated with alcohol, and washed free from ammonia with water.

Hair (Human)—A preparation previously analyzed for bases in this laboratory by the silver precipitation method (26).

Wool (Sheep)—A commercial preparation for surgical dressings, previously analyzed in this laboratory (28); extracted with ether before hydrolysis.

Silk Fibroin—A preparation of commercially de-gummed silk from a uniform strain of cocoons; in skeins, a gift of Professor T. B. Johnson of Yale University to the late Dr. Osborne in 1923.

DISCUSSION

The data in Table I give the mean of four or more determinations with the standard error. The uncertainty is in most cases less than 1 per cent of the proportion of arginine found.

The group of seed globulins was examined in the hope of finding an even richer source of arginine than edestin.[1] Kossel and Gross analyzed a preparation which they referred to as *Arachisprotein*, finding 30.8 per cent of the nitrogen to be arginine nitrogen. If their material possessed the same nitrogen content as authentic arachin, this result would be the equivalent of 17.5 per cent of arginine, the highest proportion that has been reported in any protein except the protamines and histones. The present results, however, confirmed the order of magnitude of the considerably lower figure of Johns and Jones, and the nature of the material analyzed by Kossel and Gross is left uncertain.

The present value of 2.57 per cent of arginine in gliadin is very close to Hunter and Dauphinee's (4) figure 2.64 per cent, obtained by the arginase method, and is identical with that of Johnson and Coghill (14) obtained by the Van Slyke method. Van Slyke himself (5), however, obtained the much higher proportion of 3.13 per cent. The value 2.92 per cent, obtained by Osborne and Leavenworth (12) by the Kossel and Kutscher procedure, is the highest of six determinations, the average of which was 2.69 per cent. Nevertheless Graff, Maculla, and Graff's (13) recent value by a micro arginase method is identical with this high figure. Accordingly the yield of arginine from gliadin is still somewhat in doubt. The present result is possibly a little too low.

A previous value for zein was confirmed but this, like that for gliadin, may be a little too low (see Vickery (15)).

Casein was repeatedly analyzed and the final average of 3.72 per cent of arginine is identical with one of the two values (3.72 and 3.97 per cent) secured by Vickery and White (16) by analysis of the same preparation by the silver precipitation method. Hunter and Dauphinee's arginase value and Van Slyke's own de-

[1] Krishnan and Krishnaswamy (*Biochem. J.*, 33, 1284 (1939)) have recently found that the crystalline globulin of watermelon seeds contains approximately 30 per cent of its nitrogen as arginine nitrogen. This is the equivalent of a yield of 17.3 per cent of arginine.

termination agree quite well with this. In view of the well known inhomogeneity of casein it is probable that close agreement among different workers is scarcely to be expected and more exhaustive study is not warranted until demonstrably homogeneous fractions become available. An example of wide variations of apparent arginine yield from different casein preparations is to be found in the recent work of Thomas, Ingalls, and Luck (33).

Horse hemoglobin was given particular attention, since it is the most thoroughly characterized of the protein preparations available. Two different lots of crystalline material were analyzed with substantially identical results. The average value of 3.59 per cent corresponds exactly with that to be expected if 1 molecule of hemoglobin of weight 68,000 (34) yields 14 molecules of arginine. A molecular weight of 66,000, based on Zinoffsky's (35) iron and sulfur analysis, would require 3.69 per cent of arginine for 14 molecules, which is probably within the limits of accuracy of the data concerned in the calculation.

The γ-pseudoglobulin samples prepared from horse serum (four in all, each analyzed separately) represent material which had been demonstrated to be homogeneous by the electrophoresis method. No arginine determinations in serum protein preparations of comparable homogeneity have previously been published.

The result for cattle blood fibrin confirms the highest of Bergmann and Niemann's (22) values obtained by the flavianic acid method of Kossel and Gross, and differs but little from that of Hunter and Dauphinee obtained by the arginase method. The result of the gelatin analysis is also very close to that of Hunter and Dauphinee.

It is difficult to calculate the molecular relationship in egg albumin because of the wide range in the values that have been assigned to the molecular weight of this protein. If Svedberg's (34) measurements of 40,500 or 43,800 be taken, the present result corresponds closely to that calculated respectively for 13 or for 14 moles per molecule.

The values for human hair and for wool, appreciably higher than those earlier obtained by the silver precipitation method, illustrate the advantage of the present procedure for the analysis of proteins that contain unusually high proportions of cystine. A previous value for silk fibroin was confirmed but is possibly a little low.

A comparison of the arginine values of Kossel and Gross (1) with the results of the present analyses shows little agreement. These authors did not describe the method they employed to purify the arginine monoflavianate secured by direct precipitation from the protein hydrolysates, save to state that it was heated with a considerable volume of very dilute flavianic acid solution. Experience with the material precipitated directly from hydrolysates of edestin under conditions that favor the formation of the monoflavianate indicated that these precipitates often weigh more than would be expected from the present arginine value. Attempts to purify them by treatment with water or with dilute flavianic acid solution did not give satisfactory results.

There is also little agreement between the present values and those secured by Fürth and Deutschberger (19) who, in most cases, weighed arginine monoflavianate precipitated from a base fraction obtained with phosphotungstic acid.

EXPERIMENTAL

Hydrolysis of Protein The quantity of protein required depends upon its richness in arginine and the number of replicate determinations contemplated. The individual samples should preferably give 0.5 gm. or more of arginine monoflavianate, and this is accomplished if about 5 gm. of a protein of low arginine content, such as casein, are taken; from 2 to 3 gm. of a seed globulin are adequate. The data in Table I were obtained by the hydrolysis in each case of from 20 to 25 gm. (for silk 43 gm.) of the protein with 500 ml. of 20 per cent hydrochloric acid for 24 hours at boiling temperature. The hydrolysate is repeatedly evaporated to a syrup *in vacuo* and then made to 250 ml.; 1 ml. aliquots are removed for nitrogen determination. The quantity of protein carried on is calculated from the nitrogen content given for each protein in Table I. These data were mostly taken from the literature and represent the nitrogen of dry, ash-free preparations of the highest purity.

The solution is diluted, boiled with about 5 gm. of norit, and the norit is filtered off and extracted twice with boiling water. The nearly colorless hydrolysate and the washings are then filtered and brought to 250 ml., and 50 ml. aliquots are taken for each of four arginine determinations. For the analysis of single small samples the quantities are appropriately scaled down, the

aim being to obtain a final solution of approximately 10 per cent concentration.

Precipitation of Diflavianate—The quantity of flavianic acid required is calculated from the best available estimate of the arginine content (1 gm. of arginine requires 1.805 gm. of flavianic acid for 1 mole) and from 4 to 5 moles are added in substance at *room temperature*.[2] The samples are then placed in the refrigerator for 4 days, being thoroughly stirred at least once each day. Under these conditions, the product that separates consists chiefly of the pale yellow needles of the diflavianate of arginine, occasionally mixed with a little of the orange-yellow monoflavianate in the form of nodular masses of plates. Minor quantities of ammonium flavianate and probably of the flavianates of other amino acids may also be present.

The precipitate from each sample is filtered on a small sintered glass funnel (or crucible) and washed two or three times (about 30 ml. in all) with water saturated at room temperature with arginine monoflavianate.[3] During this operation much of the diflavianate is transformed into monoflavianate (see below), and the fluid that runs through is highly colored with flavianic acid, but no loss of arginine has been detected. Ammonium flavianate and the flavianates of other amino acids if present are largely removed.

Precipitation of Monoflavianate—The funnel is attached to a 150 ml. suction flask and the precipitate is stirred with a little hot water; 5 N ammonium hydroxide is added drop by drop from a 1 ml. pipette graduated in 0.1 ml., and the precipitate is stirred until all is in solution. The *minimal* necessary amount of reagent is used and, at the end, the solution should have a barely detectable odor of ammonia. It is then gently drawn through the sintered glass which is finally washed colorless with hot water, if necessary with the aid of a trace of ammonia. Loss from ebullition in the filter flask must be carefully guarded against.

The clear filtrate is washed with a little hot water into the

[2] In the silk analysis, the aliquots were diluted to 70 ml. and 10 moles of flavianic acid were used.

[3] To prepare this wash fluid, a few gm. of pure arginine monoflavianate are thoroughly shaken in a 2 liter flask of water and allowed to settle. The solution is filtered into a small wash bottle for use.

beaker used for precipitation and any crystals that escaped transfer to the funnel are dissolved; the total volume at this stage should be about 40 ml. The solution is carefully brought to a boil and 1 N sulfuric acid equivalent to or slightly in excess of the amount of 5 N ammonia used to dissolve the precipitate is added. Arginine monoflavianate usually crystallizes at once from the hot solution in plates with a highly developed golden yellow luster, and crystallization should be allowed to become complete at room temperature before the solution is stirred. After being chilled overnight, the crystals are filtered on a sintered glass crucible, washed several times with water saturated at room temperature with arginine monoflavianate and finally with a little alcohol, and are dried a few hours at 105°, cooled in a desiccator, and weighed with as little exposure to the air as possible (slightly hygroscopic when dry). The weight of arginine monoflavianate multiplied by 0.3566 gives arginine; by 0.1148, gives arginine nitrogen.

Discussion of Details of Procedure

The arginine monoflavianate obtained from the proteins studied has in each case been demonstrably analytically pure; sulfur determinations on composite samples are given in Table I, fifth column. The intense golden yellow luster is a characteristic property that, when well developed, appears to be a reliable evidence of purity. Preparations obtained under other circumstances and known to be impure seldom exhibit a pronounced luster.

The only contaminant that has been encountered is ammonium flavianate, which occasionally separates as a thin film of pale yellow needles, usually at the surface of the solution. This may happen if an excessive amount of ammonia is used to dissolve the first precipitate, or if the volume is too small. If this occurs, the sample may be warmed to 30–40° and allowed to stand at room temperature for a few hours before filtration. No significant loss of arginine has been detected when this was done. Alternatively, the samples may be washed more thoroughly with arginine flavianate solution than is usually necessary. No case has been encountered in which more than 100 ml. of wash fluid were required, and study of the loss of weight with repeated measured

volumes of wash fluid has shown that a valid correction for the total loss in the washings can readily be ascertained. It has been necessary to resort to this artifice in only one or two cases.

As an illustration of the reproducibility obtained in the analysis of edestin by various modifications of this procedure, detailed data from several experiments are shown in Table II. The diflavianate technique is definitely, although only slightly, superior with this high arginine protein. With casein and zein, no significant difference could be established. The use of the diflavianate was suggested by the behavior noted after flavianic acid had been added to the hot solution of the hydrolysate. The mother liquor, from which arginine monoflavianate had apparently separated completely, slowly deposited pale yellow needles on being chilled for several days. Examination of these showed that they consisted of arginine diflavianate, and it became evident that this salt is less soluble than the monoflavianate in the acid solution (pH 1.0 to 1.5) of the products of hydrolysis after the addition of

TABLE II

Determinations of Arginine in Edestin by Various Modifications of the Procedure

	Individual determinations	Average
	per cent	*per cent*
1st precipitation as monoflavianate from hot solution; washed with water, dissolved, and reprecipitated	16.06	
	15.96	
	16.27	
	16.11	16.10
1st precipitation as monoflavianate; washed with saturated arginine flavianate solution, dissolved, and reprecipitated	16.50	
	16.57	
	16.54	
	16.47	16.52
1st precipitation as diflavianate	16.77	
	16.79	
	16.90	
	16.77	16.81
1st precipitation as diflavianate; preparation from Osborne collection	16.70	
	16.79	
	16.73	
	16.64	16.72

a large excess of the reagent. Clearly, therefore, the diflavianate is to be preferred for the *preliminary* separation of the arginine. An analogous case of relative solubility of the two salts of arginine has recently been pointed out by Bergmann and Stein (36) for the naphthalene-β-sulfonates (nasylates).

A further advantage of the diflavianate technique became evident during work with casein. In the hope that more complete separation of arginine monoflavianate might be achieved if the excess of mineral acid were neutralized with ammonia, samples of hydrolysate were brought to about pH 4 before the reagent was added. Under these circumstances tyrosine became a troublesome contaminant. Nevertheless, a value of 3.74 per cent of arginine was obtained as the average of four determinations. A repetition gave 3.71 per cent, and the diflavianate technique gave 3.70 per cent. It was concluded that the latter method was to be preferred on grounds of convenience, although there is little to choose on grounds of precision with a protein of low arginine content.

The time required for complete separation of the diflavianate was also tested with casein. The yield of arginine, after crystallization had proceeded for 24 hours in the refrigerator, was 3.36 per cent; after 48 hours, 3.57 per cent; after 5 days, 3.70 per cent.

The necessity for a large excess of reagent was evident from experiments with edestin in which the formation of the monoflavianate was promoted by conducting the precipitation in hot solution and subsequently allowing crystallization to proceed for 3 days in the cold. Under these circumstances, the largest possible excess of flavianic acid was present in the mother liquor to favor the formation of the diflavianate with the traces of arginine that remained in solution. When 1 mole of flavianic acid was added, 14.4 per cent of arginine was isolated; with 1.5 moles, 15.9 per cent; and with 2 moles, 16.3 per cent. The use of 4 to 5 moles, the latter amount with proteins of low arginine content, is clearly desirable when the diflavianate is allowed to form from the beginning.

Tests of the recovery of arginine after precipitation as the monoflavianate were made by treating 0.3738 gm. of arginine (as monohydrochloride) dissolved in 30 ml. of water with 2 moles of flavianic acid at boiling temperature. The crops of crystals

obtained from the chilled solution contained 0.3722 and 0.3736 gm. of arginine in two experiments, an average yield of 99.8 per cent. A similar quantity of arginine was added to 30 ml. of an approximately 10 per cent solution of the products of hydrolysis of edestin from which arginine and the excess of flavianic acid had previously been removed. On the addition of flavianic acid to the hot solution, arginine monoflavianate separated slowly and the solution was heated, after having been chilled overnight, to insure that all diflavianate had been decomposed. After being again chilled overnight, the crystals contained 0.3734 and 0.3751 gm. of arginine in two experiments, an average yield of 100.1 per cent. No significant loss of arginine is thus to be anticipated in the manipulations.

Incidental observations on the solubility of arginine monoflavianate showed that samples of about 1 gm., washed at room temperature (about 24°) on the crucibles with 100 ml. of 1 per cent aqueous flavianic acid, lost on the average 2.5 mg., which is barely significant in work on the scale of these experiments. When washed with 100 ml. of water, the loss was somewhat variable but averaged 18 mg. Accordingly the use of water as a wash fluid was discarded.

When samples were washed with 100 ml. of water saturated with arginine monoflavianate at room temperature, the average loss observed was close to 2.4 mg. Theoretically there should be no loss under these conditions and doubtless, if the experiments were carried out at a controlled constant temperature, this would be achieved. The point, however, was to establish the behavior under convenient working conditions and the result showed that the usual washing operation with from 25 to 50 ml. of wash fluid can be conducted without significant error.

The solubility of arginine monoflavianate, determined by evaporating 100 ml. of saturated aqueous solution in platinum dishes, was found to be 20.0 mg. at 24° and 11.8 mg. at 7.5°. Kossel and Gross gave the figure 17.7 mg. at 19°. The present observations are not offered as exact solubility determinations; they serve merely to establish the order of magnitude, and the results suggest the desirability, if attempts are made to conduct the present procedure on a semimicro scale, of working at a controlled and low temperature.

The solubility in absolute alcohol is too low to be estimated easily by direct weighing. The color of a saturated solution at room temperature is much paler than that of a saturated aqueous solution diluted 10-fold, and approximate determinations with the Pulfrich photometer suggested an order of magnitude considerably less than 1 mg. in 100 ml. Accordingly alcohol may be used as a wash fluid without significant loss.

The solubility of arginine monoflavianate is much depressed in the presence of excess flavianic acid. The preliminary precipitation of the diflavianate, which takes place in the presence of an approximately 10 per cent solution of mixed amino acids, is rendered as complete as possible by the use of a large excess of free flavianic acid. The washing of this compound removes much of the 2nd molecule of combined flavianic acid (see below), but is deliberately stopped before this decomposition is complete. Thus the reprecipitation as monoflavianate normally takes place in the presence of excess flavianic acid. In certain cases in which this has not been obvious from the appearance of the precipitate, a little flavianic acid has been added to the ammoniacal solution, although no particular advantage therefrom has been demonstrated. The risk that ammonium flavianate may separate is definitely increased by this procedure and it may therefore defeat its own end by making a more extensive final washing necessary.

Arginine Diflavianate—No exhaustive study of the properties of arginine diflavianate has been attempted, but a few incidental observations may serve to illustrate its value as a means for the isolation of arginine. The possibility that this substance may form in acid solution apparently escaped the attention of Kossel and Gross and of Fürth and Deutschberger, and its casual presence may help to account for the failure of these investigators to secure more reliable arginine determinations in proteins.

The first reference to the substance is in a foot-note to a paper from this laboratory (37) in 1927 and it was shortly afterwards studied briefly by Cox (38) who found that it separates, frequently together with the monoflavianate, when recrystallization of monoflavianate is attempted from strongly acid solutions. The simplest method to prepare a specimen is to dissolve 3 gm. of the monoflavianate in 25 ml. of boiling 10 per cent hydrochloric acid. On being cooled, the diflavianate separates as pale yellow needles

in radiating masses. It may be filtered with suction on sintered glass and washed with a very little cold 10 per cent hydrochloric acid without apparent decomposition. It may be dried *in vacuo* over lime, although this is slow. It does not appear to decompose if nearly dry material is heated at 105° in the oven. The yield (no evidence of water of crystallization was encountered) varied from 96 to 98 per cent of that calculated on the assumption that 2 moles of arginine monoflavianate yield 1 mole of arginine diflavianate and 1 of arginine in acid solution. The arginine in the filtrate was recovered as monoflavianate, the yield being from 98 to 100.2 per cent of that calculated.

Somewhat less satisfactory results were obtained if the calculated amount of flavianic acid and of arginine monoflavianate were dissolved together in hydrochloric acid, and, when a substantial excess of flavianic acid was used, the product was contaminated with this substance.

On being heated, the diflavianate shrinks and softens in a characteristic manner at about 160°, as described by Cox, but appears to become solid again at 180°. Decomposition is evident from about 220° and pronounced gas evolution occurs at or near 228°. Cox's observation that the substance decomposes at 245°, uncorrected, could not be confirmed. The temperatures given were observed with a short stem thermometer.

Arginine diflavianate loses flavianic acid when treated with cold alcohol or cold water and is instantly decomposed by hot water. A dry sample (2.4327 gm.) was heated with 50 ml. of water, cooled, and the monoflavianate was recovered. It weighed 1.4800 gm., a yield of 100.0 per cent of that calculated. The filtrate was treated with an excess of arginine monohydrochloride. The arginine monoflavianate obtained weighed 1.4672 gm. and thus contained 0.9441 gm. of flavianic acid or 99.1 per cent of that calculated. The decomposition of arginine diflavianate by hot water is therefore strictly quantitative.

Another sample (2.4267 gm.) was washed on a crucible at room temperature with 100 ml. of saturated arginine monoflavianate solution. The color of the clear filtrate was much intensified and, by the addition of an excess of arginine hydrochloride, the flavianic acid it contained was recovered. The weight of the arginine monoflavianate obtained, corrected for the approxi-

mately 20 mg. present in solution in the wash fluid, was equivalent to 0.8687 gm. of flavianic acid or a little more than 91 per cent of the 2nd mole of flavianic acid present in the sample of arginine diflavianate taken. Thus this substance is extensively decomposed by being washed at room temperature with a considerable volume of saturated aqueous arginine monoflavianate solution. An important step in the analytical procedure depends on this observation. Complete decomposition of the diflavianate during washing would be undesirable, since some excess of flavianic acid is needed during the recrystallization in order to repress solubility. The technique of washing described achieves this end more or less automatically.

The filtrate from the experiment last mentioned, from which flavianic acid had been precipitated with an excess of arginine, was nearly colorless and a rough colorimetric estimate showed that it contained much less than 1 mg. of arginine monoflavianate per 100 ml. The solubility of this substance is thus as markedly repressed by the presence of an excess of arginine as it is by an excess of flavianic acid.

No attempt was made to investigate the exact solubility of arginine diflavianate. The substance apparently cannot exist in aqueous solution and is at once decomposed when warmed with quite dilute hydrochloric acid. The arginine monoflavianate redissolves at boiling temperature if 2 per cent hydrochloric acid is used, and the loss of orange color suggests that the di-salt is again formed at the higher temperature. Nevertheless the solution deposits the monoflavianate when cooled, although a little diflavianate may separate on long standing. On the other hand, if 4 per cent hydrochloric acid is used, there is no detectable decomposition (orange color) when the sample is warmed until dissolved. When cooled, the solution deposits mainly yellow needles of diflavianate, although minor amounts of the orange monoflavianate usually accompany it. This has been occasionally observed even with 10 per cent acid.

A thorough study of the solubility of arginine diflavianate would involve examination of the equilibrium relationships at different temperatures between arginine, flavianic acid, and mineral acid and is beyond the scope of the present investigation. It suffices to point out that the solubility of this substance must be ex-

tremely low in the 10 per cent solution of mixed amino acids at a reaction in the vicinity of pH 1 to 1.5 when a 100 per cent or larger excess of flavianic acid is added. This is clear, since the solubility is obviously less than that of the monoflavianate and this substance can be almost quantitatively recovered under these conditions.

One further comment may be added. Bergmann and Stein (39) have recently pointed out that the determination of amino acids by precipitation in the form of a salt can seldom be completely satisfactory because of the residual solubility in the mother liquor, this solubility being, in the particular case of protein analysis, a complex and quite unknown function of the composition of the mother liquor. Efforts to minimize solubility losses by concentration are defeated by the increased solvent power of the more concentrated amino acid solutions. They accordingly propose the abandonment of the use of highly insoluble salts and substitute reagents that give somewhat sparingly soluble products. By calculation from the results of a series of solubility determinations in the presence of known amounts of the reagent it becomes possible to ascertain the quantity of the given amino acid that remains in solution and thus to secure analytical figures of a high degree of probable accuracy. The method holds forth promise of being exceedingly valuable. In the present case, however, it seems that the residual solubility of arginine diflavianate is so small that the error becomes in fact negligible when an adequate quantity of protein is taken. Fortunately this quantity is within reasonable bounds for many important proteins and there seems little doubt that a fair degree of precision can be obtained by suitable modification of the technique for application to even smaller amounts.

SUMMARY

A method to determine arginine in hydrolysates of proteins is described, which depends upon the insolubility of arginine diflavianate in the acid solution of amino acids. This substance separates slowly in the cold as pale yellow needles. Purification is effected by solution in ammonia and reprecipitation at slightly acid reaction from hot solution as the monoflavianate, which separates as orange-yellow plates with a highly developed golden

yellow luster. Analysis of a representative group of proteins has given results usually a little higher than those previously obtained by the silver precipitation method. From 3 to 5 gm. of protein are required for each determination and the results are highly reproducible and apparently reliable.

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A METHOD FOR THE DETERMINATION OF SMALL AMOUNTS OF POTASSIUM*

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Our studies on the potassium content of human blood serum (1) required an analytical procedure sufficiently fine to disclose slight changes in the potassium level, a method which equals in accuracy but is less time-consuming than the platinic chloride method. Of the available micromethods which do not employ platinum for precipitation, that of Breh and Gaebler (2) and its modification by Truszkowski and Zwemer (3) appeared preferable. These authors made use of the reaction of silver cobaltinitrite with potassium, which was studied and described by Burgess and Kamm (4) in 1912 as a qualitative test for potassium. The reaction product, silver potassium cobaltinitrite, is far less soluble in water than the sodium potassium cobaltinitrite compound of the widely used Kramer and Tisdall (5) procedure. Leaving out of consideration the colorimetric measurement of either cobalt (2) or nitrite (3), we resorted to the superior techniques of gasometric determination of the nitrite as described by Kramer and Gittleman (6) and the titrimetric determination of silver by the use of the Volhard procedure.

In a systematic study of the procedure of Breh and Gaebler and its modification, the recovery of potassium from known solutions showed errors ranging from 5 to 20 per cent. We found that the source of these errors lay in the variability of composition of the precipitated silver potassium cobaltinitrite compound. Burgess and Kamm pointed out that when a solution of a potassium salt

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is added to a solution of silver cobaltinitrite, the reaction proceeds as follows:[1]

$$Ag_3Co(NO_2)_6 \xrightarrow{K} Ag_2KCo(NO_2)_6 \xrightarrow{K} AgK_2Co(NO_2)_6$$

On this basis it is to be assumed that the equilibrium between the individual compounds forming the reaction product is affected by the relative concentrations of potassium and silver participating in the reaction, and that in consequence the qualitative reaction of Burgess and Kamm cannot be used for quantitative purposes unless standard conditions which insure the constant composition of the reaction product can be established. Full control of the silver concentration in the reaction mixture being of such importance, we used in our equilibrium studies pure sodium cobaltinitrite, prepared according to Treadwell (7) with slight modifications, instead of the crude mixture prepared by pouring together sodium nitrite, cobalt nitrate, and acetic acid, as used by other workers (2, 3). Those who used such mixtures disregarded the warnings of Burgess and Kamm concerning the formation of silver nitrite in the presence of excess nitrite ions, and also failed to take into consideration the adverse influence of excess acid upon the stability of the precipitated reaction product.

Equilibrium Studies

Effect of Silver Concentration—In this series of experiments we studied the effect of the amount of the silver that participates in the reaction upon the composition of the reaction product. The pure potassium sulfate employed was put up in 0.22276 per cent stock solution, containing 1 mg. of potassium per cc.; dilutions were made as needed in these and subsequent experiments. The following reagents[2] were required.

1. Sodium cobaltinitrite, prepared as follows: 150 gm. of sodium nitrite are dissolved in 150 cc. of hot water; after the solution is cooled to about 40°, 50 gm. of cobalt nitrate crystals are

[1] Our equilibrium studies (see Fig. 1) show that this equation does not represent the true course of the reaction. Regardless of this fact, however, our experiments bear out the assumption that the concentration of silver seriously affects the course of the reaction.

[2] We used Mallinckrodt A. R. grade reagents, which were consistently satisfactory.

added with vigorous stirring until dissolved. Following this, 50 cc. of 50 per cent acetic acid are added in approximately 2 cc. portions, with whirling after each addition. Half-way through and at the end, the flask is stoppered and vigorously shaken. The yellow-brown precipitate is filtered off and discarded. After aeration for 45 minutes with a rapid current of air, the filtrate is allowed to stand in the ice box overnight. The supernatant liquid is then passed through a dry filter paper and the brown precipitate is discarded. To the filtrate, about 200 cc. of 95 per cent alcohol are added in small portions with vigorous agitation. After 30 minutes standing the precipitate is filtered off on a Buchner funnel with suction and is washed with four successive 25 cc. portions of 95 per cent alcohol, followed by three washings with pure ether. The precipitate is then placed in a vacuum desiccator, allowed to dry, ground in an agate mortar, and stored in a glass-stoppered brown bottle. The dry salt is stable.

2. Sodium hydroxide, 10 N and 1.3 N.
3. Urea, 10 per cent solution.
4. Sulfuric acid, 8 N.
5. Nitric acid, 1:2 dilution (1 volume of HNO_3 and 1 volume of H_2O).
6. Potassium thiocyanate, 0.0025 M. The dilution is prepared from 0.1 M stock solution and is not used for more than 2 days.
7. Ferric ammonium sulfate, 20 per cent solution containing 3 cc. of nitric acid per 100 cc.

Precipitation of Silver Potassium Cobaltinitrite—Into a series of 15 cc. Pyrex centrifuge tubes[3] were measured 5 cc. portions of a potassium sulfate solution containing 0.1 mg. of potassium, and to each was added a 0.5 cc. portion of a silver nitrate solution of a different concentration (see Fig. 1). The contents of the tubes were thoroughly mixed with the aid of fine glass rods, and the tubes and rods placed in a rack (an individually spaced wire basket) and immersed in a water bath at 18–20°. When the tubes

[3] The 15 cc. Pyrex centrifuge tubes must be prepared for use by treatment with hot chromic-sulfuric acid solution and thorough rinsing with tap water and then with ammonia-free distilled water. The tubes are inverted to drain on a clean sheet of filter paper; they must drain without the breaking of the film of water or else the acid treatment is to be repeated. No ammonia must be used in proximity to the work bench.

had assumed the temperature of the bath, there was added, dropwise with stirring, 0.5 cc. of a freshly prepared 12.5 per cent solution of sodium cobaltinitrite (temperature 18–20°). The tubes were allowed to stand in the water bath for from 10 to 20 minutes and then centrifuged for 10 minutes at 3000 R.P.M.[4] The supernatant fluid was removed by suction, a finely drawn glass tube with the end curved upward being used. At all times when the tubes were not in the centrifuge or in manipulation, they were kept in the water bath. Three washings with 5 cc. portions of distilled water (temperature 18–20°) were performed in the following manner. The tube was held in a slanting position (at an angle of about 25° to the vertical) and rotated while about 1 cc. of the first portion of water was allowed to run dropwise and slowly down the sides of the tube; the rest was allowed to run in rapidly. This technique insured thorough washing of the entire surface, leaving the precipitate undisturbed. Keeping the precipitate intact is essential in order to avoid slight yet measurable losses; for, although the compound is insoluble in water, some of it goes into a finely dispersed state and is lost in the course of repeated washings if the precipitate is stirred up. After each washing the tubes were centrifuged before the supernatant fluid was removed. After the last washing the tubes were covered with clean pieces of tin-foil and kept in an ice box until they were analyzed.

Manometric Determination of Nitrite—Into the tubes containing the silver potassium cobaltinitrite precipitate, 1 cc. of 1.3 N sodium hydroxide was introduced, and the contents mixed with fine glass rods. The tubes and rods were placed in a boiling water bath for from 8 to 10 minutes. The yellow color disappeared and a precipitate consisting of silver oxide and cobaltous hydroxide appeared at this operation. The solution was quantitatively transferred into the cup of the Van Slyke manometric apparatus by rinsing the tube with five 0.5 cc. portions of 10 per cent urea solution. It is, of course, important that the tube be well washed and the washings carefully transferred. The solution was lowered into the extraction chamber until the level of

[4] Several workers (2, 3) have reported difficulties with the precipitates floating and sticking to the sides of the tubes. With the technique described here, we have not encountered these difficulties.

the fluid in the cup was just above the capillary. The cup was then washed with 0.5 cc. of 10 per cent urea solution, which was also lowered into the extraction chamber. From this point on, the only deviation in our procedure from that of Kramer and Gittleman was that we deaerated the solution in the extraction chamber before adding the 1 cc. of 8 N sulfuric acid. We have found that this deaeration makes a difference of from 2 to 3 mm. of pressure in our final reading. A blank analysis was performed on each batch of the reagents used in the potassium determination; with the technique and the reagents specified, the blank value for the mixed reagents was found to be the same as the blank obtained on 1 cc. of 1.3 N sodium hydroxide alone.

Titrimetric Determination of Silver—For the estimation of the silver content, 0.5 cc. of 1:2 nitric acid was measured into the tube containing the silver potassium cobaltrinitrite precipitate, and the tube was placed in a boiling water bath for 5 minutes. If at the end of 5 minutes the precipitate was not in solution, the tube was carefully agitated. The tube and contents were allowed to cool and 1 drop of 20 per cent ferric ammonium sulfate was added. The solution then was titrated with 0.0025 M potassium thiocyanate to the appearance of the faintest, but permanent, pink color, a fine tipped microburette of 0.01 cc. graduations being used. In the small volume used the end-point is very sharp, so that by splitting drops near the end-point one can duplicate titrations with a maximum error of 0.01 cc.

The results of these experiments are presented graphically in Fig. 1. The lower curve shows the nitrite content of precipitates which were formed with a constant amount of potassium (0.1 mg.), but with the addition of variable amounts of silver to the reaction mixture. The lowest amount of added silver represented in the graph is a multiple of the equivalent required to precipitate the potassium completely, and we have ascertained that all of the potassium present actually is precipitated in every instance. Yet, as may be seen, the nitrite content rises steeply as the added silver is increased. Thus, for example, increment of a single mg. in the amount of silver can cause well over 10 per cent rise in the nitrite content of the reaction product. As shown in the curve, however, this rise persists only until the amount of the added silver approaches 10 mg. At this point the curve breaks

and the nitrite content of the precipitate remains constant until the amount of added silver exceeds 15 mg. Beyond this range the ratio again abruptly rises in favor of the nitrite content. This, we observed, is not due so much to a change in the composition of the reaction product as to the formation of insoluble silver nitrite. If the latter process is prevented by increasing the reaction temperature, it can be demonstrated that even with

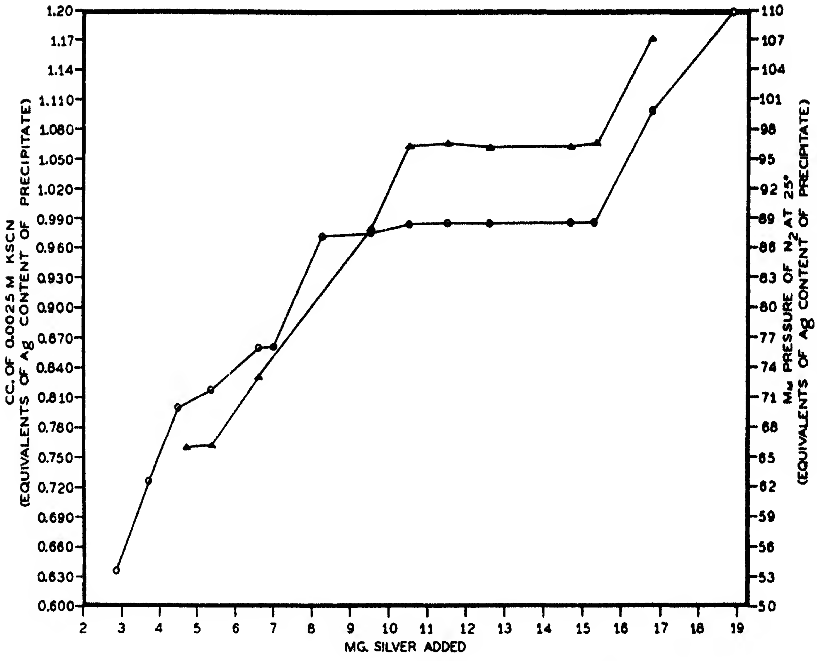

FIG. 1. Effect of variations in silver concentration upon the composition of silver potassium cobaltinitrite precipitates.

higher silver concentrations reaction products are formed in which the nitrite content rises but slowly. We adhere to the lower temperature, however, for two reasons. One is that the precipitate has a more favorable consistency for a perfect separation by centrifugation than at higher temperatures; the other is that at higher temperatures the sodium cobaltinitrite reagent decomposes so that during the ensuing operations simple nitrite is formed, enhancing the undesirable production of silver nitrite.

The upper curve in Fig. 1 represents the silver content of reaction products that were precipitated under the same conditions as prevailed in the experiments carried out for the determination of the nitrite content. As may be noted, the two curves run fairly parallel. The main interest attaches to the horizontal sections of both curves, which demonstrate that when the amount of silver reacting with 0.1 mg. of potassium ranges from 10 to 15

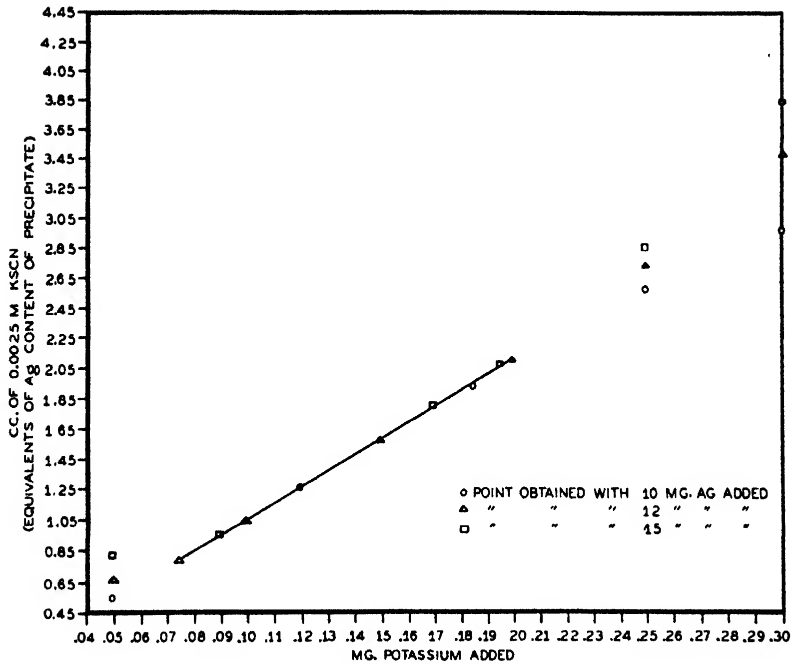

FIG. 2 Effect of variations in potassium concentrations upon the composition of silver potassium cobaltinitrite precipitates.

mg., both the nitrite and silver contents of the reaction product maintain a constant ratio to the potassium.

Effect of Potassium Concentration—In the following experiments we maintained the amount of the added silver as a constant. In three series of experiments, 10, 12, and 15 mg. of silver respectively were employed as constant quantities. These are the silver concentrations located at the beginning, the middle, and the end of the horizontal zones of both curves in Fig. 1. Other

experimental conditions were identical with those observed in the preceding experiments. The results show that with all three silver concentrations employed in these experiments, both the nitrite and the silver contents of the reaction products were in direct linear proportionality with the amount of potassium, so long as the latter was not less than 0.075 and not more than 0.20 mg. On either side of this range, however, changes in the silver concentration entailed variable degrees of deviation from proportionality. These findings are presented in Fig. 2.

From the experiments described, the following information is derived. The data represented in Figs. 1 and 2 show that under well defined experimental conditions, *one obtains silver potassium cobaltinitrite precipitates which are of a constant composition as regards the quantitative relationship between their potassium, silver, and nitrite contents.* The two salient points among these conditions are that the quantity of the potassium be no less than 0.075 and no more than 0.20 mg., while the amount of silver be confined within the range of 10 to 15 mg.

Estimation of Potassium in Blood Serum

Blood samples for the estimation of serum potassium must be taken with the observance of strict precautions for avoiding the passage of potassium from corpuscles to serum. The use of a syringe is to be avoided because the necessary slight suction involved in its use almost invariably causes slight hemolysis. We allow the blood to flow directly from a medium bore needle, which has been inserted into an arm vein, into a thoroughly cleaned, 18 × 150 mm., Pyrex test-tube. The blood is not allowed to stand in the tube for longer than 20 minutes, as there occurs in clotted blood a slow but appreciable transference of potassium from corpuscles to serum. The tube is then centrifuged and the serum is carefully drawn off by means of a clean pipette with a rubber bulb attachment. The serum is stored in a clean and tightly stoppered Pyrex test-tube until it is to be analyzed.

Human serum contains on an average about 20 mg. of potassium per 100 cc. It may be noted that the conditions we studied were geared to the estimation of potassium in 0.5 cc. of serum, so that the useful range that was established extends from the

minimum value of 15 to a maximum of 40 mg. of potassium per 100 cc. of serum, a range that covers most abnormal and experimental conditions. For extreme instances, in which the serum potassium is below 15 or above 40 mg. per cent, 1 cc. or 0.25 cc. of serum respectively is to be used.

The wet or dry ashing procedures for serum are tedious and time-consuming; besides, one must continually be on guard against contamination and loss by volatilization. The use of a tungstic acid filtrate of serum, as employed by other workers (2, 3), is objectionable, firstly, because tungstate ions remove variable amounts of added silver as insoluble silver tungstate, and secondly, because the acidity of tungstate filtrates[5] adversely affects the stability of the silver potassium cobaltinitrite precipitate. By using the cupric sulfate-sodium tungstate method of protein precipitation (8) with a slight modification, we obtain a filtrate which is free of tungstate ions and at the same time is not too acid for our purpose (pH 5.2 to 5.4).

It is important to remove the chloride ions from the serum in order to avoid the formation of silver chloride, which would entail a substantial change in the standard amount of silver to be added later on. Peters and Van Slyke (9) give the limits of chloride values in plasma as from 95 to 110 milliequivalents, which require from 10 to 11.8 mg. of silver per cc. of plasma, about one-half of our standard quantity. To a series of twenty-five normal and pathological sera we added, directly after the deproteinizing reagents, 0.8 cc. of 0.2 M silver nitrate per cc. of serum. This is more silver than is required by the chlorides, but some excess is necessary since proteins consume some silver. There was found on analysis of 5 cc. portions of the 1:10 filtrates a silver content of from 1.55 to 2.22 mg., with an average of 1.8 mg. With such variations the silver concentrations remain well within the limits established for obtaining a silver potassium cobaltinitrite precipitate of constant composition (see Fig. 1).

Precipitation of Proteins and Chlorides—For the preparation of blood filtrates free of proteins and of chlorides, the following three solutions are required, (1) cupric sulfate, 5 per cent solution,

[5] In order to attain complete precipitation of proteins from serum or plasma with the tungstate method, the reaction must be adjusted close to a pH of 3.

(2) sodium tungstate, 3 per cent solution, and (3) silver nitrate, 0.2 M solution.

With a 1 cc. Ostwald pipette ("to contain"), 1 cc. of serum is measured into a Pyrex test-tube containing 6.2 cc. of water and the contents are mixed well. 1 cc. of 3 per cent sodium tungstate is admixed; then 1 cc. of 5 per cent cupric sulfate is added, and the tube is stoppered and thoroughly shaken. Following this, 0.8 cc. of 0.2 M silver nitrate is added, the tube is again stoppered and thoroughly shaken, and the contents are filtered through a 9 cm. filter paper (No. 575 Schleicher and Schüll). It is sometimes necessary to return the first portion to the filter to obtain a perfectly clear filtrate.

If under certain exceptional experimental or pathologic conditions the serum potassium may be expected to fall below 15 mg. per cent, it becomes necessary to use 1.5 or 2 cc. of serum and to perform the precipitation at 1:7.5 or 1:5 dilution. For analysis a 5 cc. portion of this filtrate (corresponding to 0.75 or 1.0 cc. of serum respectively) is employed.

Wet Ashing of Serum—If wet ashing is preferred to the above procedure, the following reagents are required, (1) sulfuric acid, 3 N solution, (2) nitric acid, concentrated, (3) perchloric acid, 60 per cent solution, (4) methyl red indicator, 0.06 per cent solution of the sodium salt, and (5) sodium hydroxide, 1.0 N and 0.1 N solutions.

With an Ostwald pipette ("to contain"), 0.5 cc. of serum is measured into an 18 × 150 mm., Pyrex combustion test-tube containing 1 cc. of 3 N sulfuric acid; 1 cc. of concentrated nitric acid is added and the mixture is boiled to the point of charring. After the flame is removed, 0.1 cc. of 60 per cent perchloric acid is added and the heating is continued with gentle boiling; if the mixture does not become clear after 3 minutes boiling, another 0.1 cc. of perchloric acid is added. Boiling is continued until the volume of the mixture is reduced to approximately 0.2 cc. The tube is allowed to cool and the contents are quantitatively transferred into a 15 cc. Pyrex centrifuge tube by rinsing with four consecutive 1 cc. portions of water. 1 drop of methyl red indicator is added and the pH adjusted to approximately 5 with 1 N sodium hydroxide; a final adjustment is made to pH 5.2 to 5.4 with 0.1 N sodium hydroxide. The final volume of the fluid

should be approximately 5 cc. The solution of the ash is chloride-free, owing to the action of the nitric acid used in the ashing procedure.

Precipitation of Silver Potassium Cobaltinitrite—The 5 cc. of protein- and chloride-free solution prepared either by deproteinization or by wet ashing are now ready for the precipitation of the potassium. If the filtrate represents 0.5 cc. of serum (1:10 dilution), 0.5 cc. of 0.2 M silver nitrate is added. If the protein-free filtrate corresponds to 1 cc. of serum (1:5 dilution), the excess silver left in solution after the removal of the chloride is so great that it necessitates diminution of the added silver to 0.45 cc. On the contrary, for solutions obtained by the wet ashing procedure, the amount of 0.2 M silver nitrate is increased to 0.6 cc. For the rest, the technique and all the experimental conditions in this and the ensuing operations connected with the precipitation and separation of the silver potassium cobaltinitrite compound are identical with those which were followed when pure potassium solutions were used.

Manometric Determination of Nitrite—The manometric determination of nitrite in these precipitates does not differ from the procedure described for precipitates obtained from pure potassium sulfate solutions. The method of calculation is as follows: $P_{N_2} = P_1 - P_0$. (To simplify calculations we reduce all pressure readings to 298° absolute scale.) The factor by which P_{N_2} is multiplied to obtain mg. of potassium in the sample analyzed is 0.001128. This factor was obtained as follows: The silver potassium cobaltinitrite reaction product of constant composition was found to give 3.723 moles of N_2 for each equivalent of potassium. From Table 30 of Peters and Van Slyke one obtains the factor for calculating mM of N_2 per liter from analysis of 1 cc. samples in 2 cc. volume at 25°. This factor is 0.1074; $0.1074 \div 3.723 = 0.02885$; $0.02885 \times 39.1/1000 = 0.001128$.

Titrimetric Determination of Silver—The titrimetric procedure is identical with that described for precipitates obtained from pure potassium sulfate solutions. The result is calculated on the basis that the amount of silver which is precipitated with 0.1 mg. of potassium requires for its titration 1.06 cc. of 0.0025 M thiocyanate (see Fig. 1). Hence, $(T \times 0.1)/1.06$ = mg. of potassium, where T represents the titration value in cc. If the amount

of serum analyzed is 0.5 cc., then potassium per 100 cc. of serum = $(T \times 0.1 \times 100)/(1.06 \times 0.5) = 18.87 \times T$; that is to say, the titration value multiplied by the factor 18.87 gives in mg. the potassium content of 100 cc. of serum.

TABLE I

Comparative Serum Potassium Determinations by Manometric Nitrite and Titrimetric Silver Procedures

Sample No.	Potassium per 100 cc.	
	Manometric	Titrimetric
	mg.	*mg.*
1	18.9	18.7
2	18.2	18.3
3	19.5	19.3
4	24.0	23.8
5	24.5	24.1

TABLE II

Comparative Potassium Determinations on Blood Serum by Silver Potassium Cobaltinitrite Method, with 0.5 cc. of Serum, and Chloroplatinate Macromethod of MacKay and Butler, with 10 Cc. of Serum

Silver potassium cobaltinitrite method	Chloroplatinate macromethod
mg. per 100 cc.	*mg. per 100 cc.*
23.6	23.9
20.6	20.4
22.9	22.6
19.8	19.5

Accuracy of Method

Comparison of Gasometric and Titrimetric Determination—In Table I are presented comparative serum potassium values obtained by parallel determinations of the nitrite and silver content of the precipitates. As may be seen, the agreement between the two sets of results is satisfactory, so that the two procedures are practically interchangeable. For our own part we prefer the gasometric technique for its precision; the titrimetric technique, on the other hand, is more rapid and easier to perform.

Comparison with Chloroplatinate Method—The data given in

Table II show the accuracy of our method as compared with a reliable chloroplatinate macromethod as described by MacKay

TABLE III

Recovery of Potassium Added to Blood Serum (Mg. per 100 Cc. of Serum)

Potassium added	Potassium found	Potassium recovered
0	18.3	0
5	23.2	4.9
10	28.0	9.7
15	33.5	15.2
20	38.7	20.4
25	43.0	24.7

TABLE IV

Serum Potassium Content of Normal Subjects

Subject	Age	Sex	Serum potassium		Subject	Age	Sex	Serum potassium	
	yrs.		*mg. per cent*	*m.eq.*		*yrs.*		*mg. per cent*	*m.eq.*
V. P.	19	F.	20.1	5.14	T. W.	30	M.	19.6	5.01
J. F.	19	"	20.1	5.14	E. S.	21	"	19.8	5.06
A. C.	18	"	19.4	4.96	A. T.	22	F.	19.4	4.96
B. M.	18	"	18.0	4.60	B. C.	35	"	19.5	5.00
C. F.	21	"	18.5	4.73	C. W.	45	"	19.7	5.03
C. S.	18	"	19.6	5.01	E. R.	24	"	18.7	4.78
W. R.	22	M.	18.0	4.60	F. S.	24	M.	20.2	5.16
W. S.	50	"	19.7	5.03	L. W.	40	"	18.6	4.75
A. K.	25	"	20.2	5.16	H. W.	24	F.	18.1	4.62
J. D.	19	F.	18 5	4.73	A. L.	45	"	20.2	5.16
E. K.	30	"	20.6	5.26	W. I.	23	M.	19.3	4.93
H. W.	19	"	18.0	4.60	A. G.	21	"	18.4	4.70
M. S.	55	M.	18.2	4.65	C. B.	26	F.	18.5	4.73
D. E.	27	"	19.1	4.88	G. S.	33	M.	18.7	4.78
A. H.	19	F.	18 2	4.65	B. L.	19	F.	19.3	4.93
M. B.	19	"	18.3	4.68	F. W.	38	"	18.9	4.83
B. T.	20	"	19.0	4.85	E. D.	32	"	18.9	4.83
H. R.	21	M.	20.3	5.11	D. D.	28	"	19.0	4.85
D. N.	20	"	18.9	4.83					
M. H.	18	F.	18.4	4.70	Minimum			18.0	4.60
S. G.	42	M.	19.9	5.08	Maximum			20.6	5.26
D. C.	28	"	19.4	4.96	Average.			19.3	4.93

and Butler (10). In this group of determinations we used the gasometric technique.

Recovery Experiments—In a procedure which involves deproteinization or ashing, the recovery of known amounts of added potassium is evidence that no losses have occurred in these operations. As shown by the figures in Table III, our method is satisfactory in this respect.

Potassium Content of Serum of Normal Human Subjects

In Table IV are recorded serum potassium values of forty healthy individuals. The subjects were members of the hospital staff—nurses and internes—on most of whom we have in the past performed various blood analyses. These data, as well as the physical condition of the subjects, justified their classification as healthy normal individuals.

As may be seen, the range of variations, 18 to 20.6 mg. per cent (average, 19.3 mg. per cent), is quite narrow. This is at variance with the wide range of values to be found in the literature, which we discussed in a recent paper (1). To cite a characteristic example, Jellinek and Looney (11), who used the analytical method of Breh and Gaebler, gave the normal variations as ranging from 14.3 to 22 mg. per cent. Since these workers used meticulous care in the selection of their subjects and other experimental conditions, we ascribe the wide range of their figures to inadequate analytical technique.

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ELECTROKINETIC ASPECTS OF SURFACE CHEMISTRY

VI. THE INTERACTION OF GELATIN WITH CASEIN AND EGG ALBUMIN AT SURFACES

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Although the investigation of the electrokinetic properties of purified proteins in solution and at surfaces has produced considerable information concerning the effects of adsorption, and the net charges and shapes of the molecules, as discussed in Papers I to V of this series (1–5) and elsewhere (6–8), little is known about film formation on surfaces when particles are placed in solutions of mixed proteins. It is this complication, however, which is frequently encountered in biological systems. For instance, it was found by Moyer (9) that latex particles from *Euphorbia* and *Asclepias* possess specific surfaces dependent on the species. Their electric mobility-pH curves fell into definite families of characteristic shapes, permitting a classification of species on the basis of the electrophoretic behavior of their latex particles in buffer solutions. This classification agreed closely with the arrangement already determined by taxonomists. Although certain of these species yielded curves whose shape and position indicated latex particles naturally coated with a single protein, the majority were of a complex form, suggesting that the surfaces in these cases had been formed by the adsorption of one protein with others or with other substances, such as sterols (10).

Selective adsorption of an active substance on particles placed in biological mixtures, such as extracts of allergenic pollens, enzymes, or hormones, and the electrokinetic behavior of these films are problems needing considerable clarification. Abramson, Sookne, and Moyer (11) have shown that quartz particles placed in dialyzed ragweed pollen extracts adsorb a protein-like con-

stituent capable of forming wheals on the skin of sensitive subjects but more work must be done before the nature of these films is made completely clear.

Furthermore, problems involving the behavior of surfaces in such complex mixtures as milk or blood serum, to name a few additional examples, frequently arise. Obviously, an analysis of the behavior of pure proteins in mixtures would be of considerable interest in the elucidation of the behavior of these systems. This and subsequent communications will attempt to throw light on the mechanism of film formation under these conditions by a comparison of the electrophoresis of microscopic particles in solutions of purified proteins and in mixtures.

In 1910 it was reported by Michaelis and Davidsohn (12) that the electrophoretic isoelectric point of oxyhemoglobin in solution was unaffected by the addition of 1 per cent serum albumin. They found that the pH of maximum flocculation in mixtures of two proteins lay between the value for either protein in the pure state when interaction occurred between proteins but that when there was no interaction two maxima were found at pH values corresponding to the maxima for the two proteins in pure solution. Later experiments by Tiselius (13), Tiselius and Horsfall (14), and others revealed that the electric mobility of a protein may be nearly unaffected by the presence of other proteins in the same solution. In mixtures of serum albumin and thymonucleic acid, it appears that the bilirubin normally attached to the albumin tends to shift its attachment to the thymonucleic acid (15) but, in general, dissolved protein molecules do not seem to interact very much unless they are of high charge and opposite sign under conditions where coacervation is encountered (16). It seems that this behavior is in keeping with experiments showing the inability of iodine to react with dissolved saponarin or starch unless a surface is present (17).

Bondy and Freundlich (18) have isolated two proteins from *Hevea* latex. Although quartz coated with either protein gave a smooth mobility curve, when quartz suspensions were added to a mixture of these proteins, the resultant curve showed clearly by its complex shape that both proteins were adsorbed. This curve agreed quite well in its general appearance with that for the natural latex particles.

It has been shown that pepsin is adsorbed by edestin (19), by fibrin (20), and by casein and denatured egg albumin (21, 22). This combination is restricted, at least in the case of egg albumin, to certain acid pH values (21).

Ultrafiltration experiments by Risse (23), using membranes impregnated with gelatin, indicate that hemoglobin is not adsorbed by gelatin, although some of his data may be complicated by the fact that he used alkaline solutions of proteins for coating his membranes, at pH values where the tendency for adsorption might have been lessened. Harvey and Danielli (24) mention that ovalbumin displaces films of pepsin on a surface trough. Bull and Neurath (25) have found that the surface denaturation of egg albumin by shaking is unaffected by the addition of gelatin to the solutions. The foregoing experiments, together with others by Langmuir and Schaefer (26), indicate (1) that one protein may replace another at a surface, (2) two proteins may be adsorbed as a mosaic, or (3) one may or may not coat the other, depending upon the proteins involved and the conditions.

Methods

Protein Preparations—An egg albumin preparation used in Paper I of this series (1) was also used here. This had been crystallized four times from sodium sulfate by the method of Kekwick and Cannan (27) and had a gold number greater than 7 (1). Solutions were dialyzed until free from sulfate. The gelatin preparation had also been used before (4). This was electrodialyzed. The casein was prepared by the method of Van Slyke and Baker (28) and electrodialyzed.[1] It was brought into solution by trituration in a mortar with a small quantity of water, followed by the addition of sufficient 0.1 N NaOH to bring the solution to pH 7 (29). All protein solutions were kept in a clear state by filtration through quantitative filter paper (Whatman, No. 42). Unless otherwise stated, a constant amount of particles was added to the protein solutions, followed by the basic constituent of the buffer, then the acid, and lastly water to produce the desired concentrations. All measurements are at a constant

[1] We are indebted to Professor L. S. Palmer and Mr. C. L. Hankinson of the Division of Agricultural Biochemistry, University of Minnesota, for this sample of casein.

ionic strength, $\mu = 0.02$. The quartz and collodion particles have been described before (1).

Electrophoresis Measurements—Two Abramson horizontal micro-electrophoresis cells were employed in accordance with techniques described in detail elsewhere (6, 30). These instruments yielded results which checked completely with data obtained on comparable substances with the moving boundary method (3, 30). All measurements have been corrected to 25° (30). pH measure-

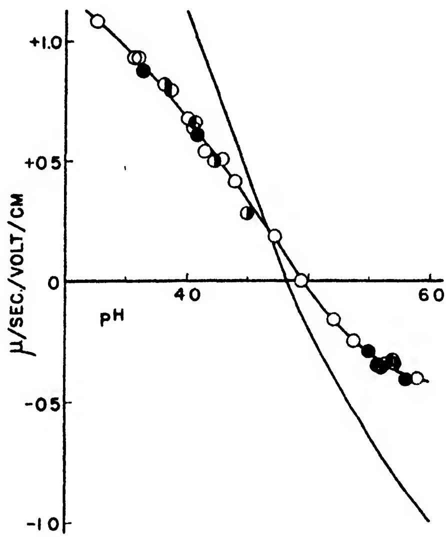

FIG. 1. The electric mobility of collodion particles in native egg albumin (smooth curve) and gelatin (O) solutions and in mixtures of the two at 25°. $\mu = 0.02$ sodium acetate. ◐, gelatin-coated collodion in albumin; ◑, albumin-coated collodion in gelatin; ●, collodion in a mixture of albumin and gelatin.

ments were determined with a quinhydrone electrode which was standardized by an equimolar mixture of 0.1 M sodium acetate-acetic acid, pH 4.64 (31).

EXPERIMENTAL

Egg Albumin and Gelatin

In Fig. 1 are shown the electric mobility curves of pure gelatin and egg albumin. The egg albumin curve is a smooth curve

drawn to fit previous data for adsorbed egg albumin on carbon, quartz, collodion, mineral oil, and glass particles (1). The gelatin curve was obtained by coating particles of quartz or collodion with gelatin solutions. Particles of collodion were next added to a 1 per cent solution of gelatin. After the coating had become complete, egg albumin was added, followed by sodium acetate, acetic acid, and lastly water to bring the final solution to a concentration of 0.02 M sodium acetate, 0.1 per cent gelatin, and 0.5

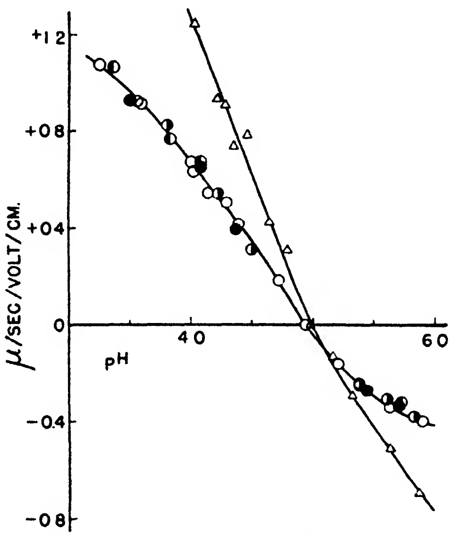

FIG. 2. The electric mobility of quartz particles in surface-denatured egg albumin (△) and gelatin (○) solutions and in mixtures of the two at 25°. μ = 0.02 sodium acetate. ◐, gelatin-coated quartz in albumin; ◑, albumin-coated quartz in gelatin; ●, quartz in a mixture of surface-denatured albumin and gelatin.

per cent egg albumin at the pH desired. These concentrations were chosen to make the results comparable with the data secured with gelatin or albumin alone and to insure complete coating. The results show that the gelatin surface was unaffected by the presence of egg albumin under these conditions. When the reverse experiment was performed, *i.e.* coating the particles first with egg albumin, the result was the same, as indicated by Fig. 1. If a mixture of these two proteins was prepared and collodion

added (followed by the buffer constituents), the resultant surface film was likewise gelatin, as evinced by the congruence of these data with the gelatin curve. Use of carbon particles or mineral oil droplets instead of collodion in this last experiment yielded the same results. The behavior of quartz in a suspension of denatured egg albumin containing considerable amounts of undenatured protein and in a mixture of this with gelatin, according to the outlines of the previous experiments, is shown in Fig. 2. Evidently denaturation before adsorption, although shifting the egg albumin mobility curve (1), does not change the effect of gelatin.

Some experiments were done on the effect of altering the order of addition of the constituents, but no differences in behavior could be noticed. Measurements made after a wait of a few minutes or several hours at room temperature gave the same result in all these cases.

Gelatin and Casein

Fig. 3 presents the data for the electric mobility of particles of quartz or collodion coated with casein in the pH range 5.8 to 7.8, where casein is soluble and the pH is not high enough to hinder adsorption. In addition, there is presented an extension of the pure gelatin curve shown in Figs. 1 and 2 up to pH 7.8, with both types of particles. Determinations were carried out in acetate and in Na_2HPO_4-KH_2PO_4 buffers at the same constant ionic strength. The results indicate that the change in ionic species has little or no influence on the position of the curves at this ionic strength.

Particles were next coated with casein and after a suitable time interval the buffer constituents were added, followed by gelatin and water to bring the concentrations to 0.02 M sodium acetate, 0.1 per cent gelatin, and 0.1 per cent casein. After several hours had elapsed, electrophoresis measurements were performed. It will be noticed (Fig. 3) that the results are nearly independent of the nature of the particle, and that the gelatin does not appear to influence the casein surface at these pH values. When particles were coated with gelatin first and then treated with casein at the same concentrations, the same time intervals being used, the results indicated by the lower dashed curve were found. It appears that the gelatin surfaces formed on these

particles were much more closely comparable with the pure gelatin surfaces than with the casein surfaces. Here too the nature of the particles seemed to have but little influence. If care was not taken to insure that the initial coating was complete by shaking the suspensions at intervals before equilibrium was reached, results tended to be less uniform.

Mixtures of casein and gelatin solutions were prepared and particles added, followed by the buffer constituents. The measurements, made after several hours wait, are drawn as squares. In this case the particle surface has a slight influence on the results,

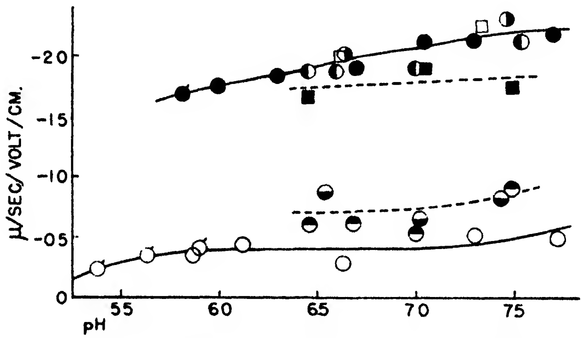

Fig. 3. The electric mobility of collodion and quartz particles in casein and gelatin solutions and in mixtures of the two at 25°. ○, gelatin alone; ●, casein alone; ◐, casein-coated quartz in gelatin; ◑, casein-coated collodion in gelatin; ⊖, gelatin-coated quartz in casein; ⊖, gelatin-coated collodion in casein; □, collodion in mixtures of casein and gelatin; ■, quartz in the same. Dashed lines have been drawn to indicate apparent shifts in behavior. Tagged circles represent measurements in acetate and untagged in phosphate buffers, $\mu = 0.02$.

for the more hydrophobic collodion particles attained a casein surface, while the quartz particles were influenced slightly by the hydrophilic gelatin. It is evident from these experiments that the first protein reaching the surface determines the final result and that there is little tendency for one of these proteins to be adsorbed by or replace the other.

Below pH 5.5, under the present conditions, casein is appreciably insoluble, down to about pH 3.5. If suspensions of casein particles are prepared by adding small amounts of casein solution to sodium acetate, followed by acetic acid to the desired pH,

finely divided casein sols are produced. The casein concentration was, in general, 0.02 to 0.05 per cent. The curve shown in Fig. 4 indicates that such particles have a steep mobility curve and an isoelectric point between pH 4.5 and 4.6, in agreement with the results of Michaelis and Pechstein (32) and Loeb (33). In Fig. 4 are shown the results for particles coated with gelatin, isoelectric at pH 4.95. Casein particles were next produced by addition of buffer to the pH value desired, followed by the addition of gelatin to a concentration of 0.1 per cent. After

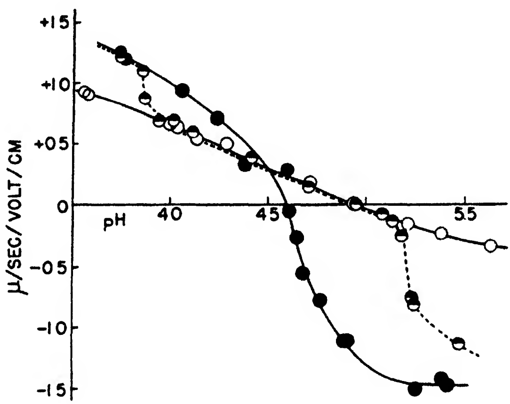

FIG. 4. The electric mobility of casein particles in the casein insolubility range (●), compared with the electric mobility of particles coated with gelatin (○) and in mixtures of the two proteins (◓) produced by the addition of gelatin to the sol after adjustment of pH. The dashed curve indicates the behavior of casein particles in contact with gelatin at these pH values.

several hours, measurements were carried out. The results are shown by the dashed line in Fig. 4. At pH values above 5.4, it is probable that the casein particles are nearly unaffected by gelatin, in view of the data in Fig. 3. Casein becomes so finely divided and soluble at pH values above 5.3 that it is exceedingly difficult to make measurements without use of dark-field illumination. The two curves, however, seem to be approaching each other. When the pH was lowered to 5.2, a sudden change ensued in the mobility and between pH 5.1 and 3.9 the casein particles were coated with gelatin, as shown by the complete agreement of

their behavior with that of gelatin. Between pH 3.85 and 3.90, the curve suddenly shifted and below this value the surface was unaffected by the presence of gelatin. The particles again became difficult to see at more acid values than those presented here. It must be emphasized that these measurements were made by subjecting the casein particles to gelatin after the final pH of the solution was established. If, for instance, casein particles were formed at pH 3.95 and gelatin was added, further addition of acid to pH 3.7 did not produce desorption of the gelatin (the ionic strength being kept constant), for the mobility of the particles so treated agreed completely with that of gelatin at this pH.

DISCUSSION

Distinction must be made between the possibility of the first protein being replaced by the second and the first protein being coated by the second. Both processes could lead to the same result so that, in these experiments on coating particles first with gelatin or albumin and then subjecting them to the other protein, one cannot, *a priori*, decide which mechanism is operative.

It is probable that gelatin cannot be adsorbed by egg albumin in solution, for the results of Bull and Neurath (25) show that gelatin is without influence on the extent or rate of surface denaturation of egg albumin by shaking, at gelatin concentrations up to the gelling point. These results indicate, furthermore, that gelatin is replaced by and cannot replace egg albumin at an air-water interface. They found, moreover, that *n*-heptyl alcohol could inhibit surface denaturation of egg albumin completely. Bull and Neurath point out that gelatin is more hydrophilic than natural egg albumin and much more so than denatured egg albumin, so that it would tend to be drawn out of the surface and into the solution. Our results show that gelatin at a solid-liquid interface is not replaced by egg albumin. If, however, egg albumin is initially present at the solid surface, gelatin will be adsorbed. It seems likely that gelatin is irreversibly adsorbed to a certain extent, for it has been found that much more uniform coating is attained on particles when they are exposed to 1 per cent gelatin followed by dilution to 0.001 per cent rather than by initial exposure to the dilute solution (4). In addition, Zsigmondy (34) found that 0.015 mg. of gelatin in 23 cc. of water did

not protect 10 cc. of gold sol, but the same amount of gelatin in 3 cc. of water added to 10 cc. of gold sol, followed by dilution to the same volume, exerted a protective action.

The electrophoresis curve for egg albumin adsorbed on inert particles is shifted upward on the pH scale parallel to that for dissolved egg albumin. Its position is intermediate between the mobility of the dissolved protein and the curve for particles of surface-denatured albumin produced by shaking. It has been suggested by Abramson, Gorin, and Moyer (8) that adsorbed egg albumin may be "partially denatured." Risse (23) has found that adsorption of egg albumin on collodion membranes is maximal at the isoelectric point but that the presence of traces of denatured material (cloudiness) produced minimal adsorption at the isoelectric point. These results and the clear difference between the adsorbed native and surface-denatured electrophoresis curves suggest that the two surfaces are not quite the same.[2] Crowther and Liebmann (36) have recently reported that the ζ potential of egg albumin after adsorption on particles is unaffected by γ irradiation, whereas it is definitely altered if the egg albumin is irradiated before addition of the particles. This also suggests that egg albumin is changed by adsorption on a solid surface.

The experiments of Lindau and Rhodius (37) indicate that at least the innermost layer of egg albumin is adsorbed irreversibly by quartz. In addition, Bull (38) has found that a solution of egg albumin in contact with a rotating smooth glass surface does not exhibit progressive denaturation. It is probable, therefore, that egg albumin is likewise held and altered to a certain extent by adhesion to the solid surface. Of the two possibilities, replacement by gelatin or double coating, the latter seems more probable. The alteration of the egg albumin by its adhesion to the

[2] Neurath and Bull (35) have suggested that a gentle washing of the particles coated with adsorbed egg albumin might remove a partial coating of undenatured protein and expose an inner layer of completely denatured material, isoelectric at pH 5.0. As stated in the original paper (1), this experiment was attempted, with the result that the particle mobilities became extremely irregular, indicating that under this treatment the surface film is no longer complete. The uniformity of the results for the native albumin, based as they are on five kinds of inert particles and five egg albumin preparations, argues against their explanation for the intermediate position of the curve.

surface might permit gelatin to adsorb on it, whereas this might not be true at an air-water interface. That particles of heat-denatured egg albumin, at least, can become coated with gelatin was shown by denaturing egg albumin with heat at pH 3.0, raising the pH until coagulation ensued, and adding some of these particles to gelatin, whereupon they assumed the mobility of the gelatin.

If one protein, *A*, is permitted to coat the surface of an inert particle which is then exposed to a second dissolved protein, *B*, without removal of the excess of *A* from the dissolved phase, the extent of adsorption of *B* should be influenced by (1) adhesive forces holding *A* to the particle, (2) possible attractive forces[3] between *B* and the particle or between *A* and *B*, (3) forces arising from the ζ potentials of the surfaces involved, (4) the hydration of the particle and the proteins, and (5) their concentrations. The possibility of attractive forces operating between the underlying surface of a particle coated with protein *A* and molecules of protein *B* in solution can be neglected because of the distances involved. If, however, protein *A* were adsorbed reversibly, such forces between the particle and *B* could play a rôle. The electrical factors will be repulsive or attractive, depending on the signs of the surfaces. Hydration will presumably act as a repulsive force (16). The net result will depend on the differences among these various factors.

When particles are added to a mixture of the two proteins, the situation is different from the above case, for in this instance there is a chance for competition between the proteins in their diffusion to the surface. In general, the final result will depend upon the factors mentioned above and also upon differences in the diffusion constants of the 2 molecules. In the case of gelatin and egg albumin, at the concentrations we employed, there appear to be the following alternatives: (1) the net attractive forces between these proteins and the particles employed are greater for gelatin than for egg albumin, (2) egg albumin reaches the surface first and is altered (possibly by dehydration) so that it attracts gelatin, or (3) gelatin is able to reach the surface first. In any event it seems likely that adsorbed gelatin exerts little or no net attraction

[3] For simplicity, no distinction has been made here between the diverse natures of various attractive forces.

on dissolved egg albumin but we cannot decide from the present data which of these three possibilities is the one of major importance here.

These results are quite different from those found with particles in the presence of casein and gelatin. For, in this latter group of experiments, when one protein had been adsorbed, the other could not affect the electric mobility to any marked extent. In a mixture of casein and gelatin, when particles are introduced, casein undoubtedly arrives at the surface before gelatin at these concentrations, for, if gelatin reached there first, the resultant mobility of the particle would presumably be that of gelatin and not casein.

The remarkable influence of pH on the adsorption of gelatin by casein particles (Fig. 4) can hardly be adequately explained by invoking the phenomenon of coacervation, for a complete coating of gelatin is attained on the basic side of the isoelectric point. At this pH both casein and gelatin are negative. In the pH range between the two isoelectric points there is a difference in the sign of charge and electrical attraction might be expected. Yet this would be an attraction between a solid particle and dissolved protein, whereas coacervation usually refers to the interaction of two sols, resulting in the separation of liquid droplets (16). No such effect was seen here. Below pH 4.6 both particle and gelatin are negative; yet gelatin is adsorbed down to pH 3.9.

It seems more likely to us that this behavior involves the interaction of specific groups on the gelatin and casein which are only available in the pH range over which coating by gelatin occurs. That the gelatin is not in reversible adsorption equilibrium with the casein is suggested by the fact that once gelatin has formed a film at pH 4.95, it does not tend to be desorbed when the pH is lowered to pH 3.7. Such irreversible phenomena presumably involve rather large changes in free energy. Since under the present conditions ζe rarely exceeds kT (6, 7), electrostatic repulsion would be expected to have but little influence in producing the present results. These interactions between gelatin and casein particles shown in Figs. 3 and 4 may be slowly reversible in some instances but no detectable progressive changes were noticed within the period of several hours after the solutions had attained apparent equilibrium.

Alexander and Bullowa (39) have reported that gelatin inhibits the acid flocculation of casein sols. We have confirmed this by experiments at 25° and, in the light of our electrophoretic results, it seems to be due to the gelatin coating which casein sols assume in the pH range on either side of the isoelectric point of casein. That gelatin will not affect casein very markedly at the pH value of milk seems probable from our experiments in Fig. 3. Alexander and Bullowa have claimed that gelatin inhibits rennet action and tends to soften the curds so formed. Palmer and Richardson (29) have found, however, that gelatin does not have this effect at 40° and Olsen (40) has shown that, while the effect noticed by Alexander may be encountered at 25°, it disappears at 37° both *in vitro* and in the stomach. These last results seem more in keeping with our findings.

SUMMARY

1. The interaction of gelatin with casein and egg albumin at surfaces has been investigated by means of electrophoresis measurements.

2. When particles of collodion are coated with gelatin or egg albumin and then placed in contact with the other protein in the dissolved state, the particles assume a gelatin surface in respect to their electric mobilities.

3. Particles of carbon, quartz, collodion, or mineral oil placed in a mixture of egg albumin and gelatin likewise became coated with gelatin.

4. These same results were found when the egg albumin solution contained surface-denatured egg albumin and the particles were quartz.

5. In the system, casein-gelatin, over the range between pH 5.8 and 7.8, the resultant surface seems to be determined by the protein which is permitted to coat the particles first. In mixtures of the two proteins to which particles of quartz or collodion were added, the casein seems to diffuse more rapidly to the surface and prevent the adsorption of gelatin for the most part.

6. Particles of casein itself, in the pH range where casein is insoluble, are not influenced much by gelatin except over a pH range which extends from pH 5.2 (slightly above the isoelectric point of gelatin) to 3.7 (a value below the isoelectric point of

casein). In this range casein becomes completely coated with gelatin.

7. The nature of the mechanisms involved in the interaction of these proteins is discussed together with their biological significance.

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ELECTROKINETIC ASPECTS OF SURFACE CHEMISTRY

VII. THE ELECTROPHORETIC BEHAVIOR OF MICROSCOPIC PARTICLES IN THE PRESENCE OF HORSE, HUMAN, OR RABBIT SERUM*

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The nature of cell surfaces when in contact with serum has been the subject of a number of investigations. As discussed by Abramson (2), these investigations extend over a wide range, including such topics as the adsorption of isoagglutinins by red cells, the surfaces of blood cells and bacteria, the Fåhraeus phenomenon, and the nature of the chylomicron surface in serum. In this field it has often been found that adsorbed films of serum protein may play an important rôle.

Tiselius (3) has recently shown by the moving boundary method of electrophoresis that horse serum is composed of a mixture of serum albumin and at least three globulins which exhibit different electric mobilities. On isolation of these proteins in the pure state by these techniques, he was able to show that their mobilities were very nearly the same as in dilute serum before separation. This indicates that serum is a mixture of well defined protein components. Stenhagen (4) has performed a similar analysis of human plasma.

Before an attempt is made to determine the nature of the surface films in those cases mentioned above in which film formation is involved, it seems necessary to investigate the composition of films produced on suspensions of inert particles, such as quartz, glass, mineral oil, or collodion, in serum from several animals and to compare these surfaces, when possible, with the films

* A preliminary account of some of these results was presented by one of us at the symposium of the Faraday Society on the "Double layer" (1).

formed on these particles when in contact with the isolated serum constituents. This may readily be done by the microscopic method of electrophoresis (2, 5). By this technique it has been found that such particles when placed in pure protein solutions adsorb the protein and assume a common electrophoretic mobility in all the cases so far investigated. This mobility agrees, in most instances, with the mobility of the dissolved protein molecules (2, 6, 7). For instance, the electric mobilities of adsorbed and dissolved serum albumin and of pseudoglobulin were found to be nearly identical (8). When, however, the protein solutions are mixtures of two components, the results vary, depending upon the proteins involved and upon the nature of the particle to a certain extent (9). We have found (9) that in some cases the acquired film appears to be a mosaic but in others its outer surface is composed almost exclusively of one or the other constituent. This appears to be due to the replacement of one adsorbed protein by the other or to the formation of a layer of the one protein on top of the other.

Davis (10) has investigated the electrophoresis of glass particles in serum and finds that these adsorb protein over the range pH 2.0 to 8.0 (the range investigated) becoming isoelectric at pH 4.5 in solutions of sufficient concentration to have produced complete coating. His work, however, was not done with the technical refinements available today. In a more complete investigation, using human and rabbit serum, Abramson (11) found that a serum concentration of approximately 1:10,000 was sufficient to produce a complete coating on the surfaces of glass, quartz, and mineral oil particles over the range pH 3.6 to 9.3, and that, once sufficient protein was present to produce a complete film, the mobility remained unchanged on further addition of serum to a concentration of 1:50. He found with human serum[1] that quartz particles and mineral oil droplets assumed an isoelectric point between pH 4.7 and 4.8 in a 1:50 serum dilution and an ionic strength of approximately 0.07. Over the range pH 3.6 to 6.0, no differences were observed between the mobilities of these two types of particles in human serum under these condi-

[1] The serum used was not explicitly stated. In a personal communication, Professor Abramson states that he used human serum in this experiment.

tions. Abramson suggests that this isoelectric point possibly indicates that "the final surface layer seems to consist chiefly of serum albumin, if the experimental isoelectric point be taken as the criterion defining the nature of the adsorbed protein. Whether a globulin surface is first formed and is subsequently covered by albumin must be decided by experiments with purified serum proteins."

Kekwick (12) has shown that two crystalline forms of horse serum albumin can be isolated by precipitation with Na_2SO_4. He finds that these components have slightly different crystal forms, those of albumin A being somewhat smaller than albumin B. Albumin A is pigmented, with a higher carbohydrate content, whereas albumin B is virtually colorless and low in carbohydrate. Both showed the same molecular weight, diffusion constant, and sedimentation constant within the limits of error. Kekwick found no difference in the electric mobilities of the two albumins in the Tiselius moving boundary instrument over the pH range 4.2 to 5.5. As pointed out before (6), his electrophoretic mobility curve, at $\mu = 0.02$, agrees very well with the moving boundary data of Tiselius and the results for adsorbed serum albumin obtained by Abramson (2) and Moyer (8).[2]

In the present publication, we shall discuss our findings on the proteins responsible for film formation on particles in serum, the effect of the buffers, and the influence of time on adsorption under these conditions. The mechanisms involved in the formation of the surface layer will form the subject of a subsequent communication.

Methods

Serum Samples—Horse, human, and rabbit sera[3] which had been obtained by clotting fresh blood were centrifuged to remove

[2] Since the completion of the investigations described here, McMeekin (13) has reported on the fractionation of serum albumin but does not give data to show the relationship of his two carbohydrate-free components to the albumins of Kekwick.

[3] We are greatly indebted to Dr. W. L. Boyd, Dr. M. H. Roepke, and Dr. Lucille M. Bishop of the Division of Veterinary Medicine, University of Minnesota, and to Dr. Eric Ponder, of the Biological Laboratory, for the serum samples used in this investigation.

traces of fibrin and blood cells. For electrophoresis, the particles were usually placed in contact with a small amount of whole serum. After a sufficient period of time had elapsed to produce complete coating, buffer solutions and water were added to give a 1:50 dilution of serum and an ionic strength of 0.1. The quartz and collodion particles were the same as those described before (14).

Protein Preparations—The procedure described by Kekwick (12) was followed to produce the albumin components. Two sets of preparations isolated from horse sera from different sources were used with no detectable difference in the results. In view of the alterations in serum noticed by Tiselius (3) after lipid extraction with alcohol-ether, only fresh untreated serum which had been centrifuged was used. Although not mentioned by Kekwick, it was found that after the crystals of albumin A and B had been separated and redissolved by his technique, adjustment to pH 4.8, before addition of salt for recrystallization, frequently produced the formation of an amorphous precipitate. We found that this precipitate could be filtered off and, on careful addition of the Na_2SO_4 to the filtrate until opalescence appeared, the albumin would crystallize. The nature of the amorphous material was not determined. Kekwick has suggested that at least one more non-dialyzable material of high carbohydrate content aside from albumins A and B must be assumed to be present, so that this material could be some of this. Our preparations of albumin A and B agreed in crystal appearance and color with those of Kekwick. They were crystallized three or four times and the final solutions were dialyzed against distilled water until no precipitate was produced by $BaCl_2$ when the water was tested.

The globulin fraction produced by the initial precipitation of the serum was redissolved and, without adjustment of pH, was reprecipitated with Na_2SO_4 a number of times. No attempt was made to separate the various globulins present in the solution. It was then dialyzed against 1 per cent NaCl until free from sulfate. Particles were coated by exposure to the stock protein solutions, followed by dilution to the desired concentration. It has been our experience that use of $(NH_4)_2SO_4$ as a crystallizing salt produces albumin crystals more easily but it has been found much more difficult to bring such preparations to the high degree of purity needed for microelectrophoresis measurements.

Electrophoresis Measurements—A horizontal Abramson micro-electrophoresis instrument was used in accordance with our usual techniques (15). The plaster of Paris plugs covering the electrodes of this instrument make possible the investigation of solutions of high ionic and protein concentration. The instrument was cleaned frequently by the use of molar NaOH followed by dilute acid and water. pH measurements were made with a quinhydrone electrode standardized against the buffers described by MacInnes, Belcher, and Shedlovsky (16). For the determination of field strength, the specific conductance of each solution was measured by a Wheatstone bridge. All data have been corrected to 25°.

EXPERIMENTAL

Horse Serum

The curves in Fig. 1 show the electrophoretic mobility of adsorbed protein constituents of horse serum. The upper curve shows the behavior of quartz or collodion particles in 0.3 per cent globulin solutions. These data are quite similar to previous data (17) secured in this manner at a concentration of 0.1 per cent but the present results are more uniform. The isoelectric point in both cases lay at pH 5.0. It is probable that one constituent of this globulin mixture is being selectively adsorbed on the particles. It would be of interest to extend these investigations by isolating the various proteins either electrophoretically (3) or by the isoelectric precipitation method of Green (18). Although Tiselius has isolated globulin components, his measurements were made at the same ionic strength but at 0°. The viscosity is the factor chiefly affected by temperature (2) but it is not the only one. If his data are recalculated to 25° by adjustment of the viscosity (although such a great adjustment is by no means free from objection), our results appear to lie closest to his for globulin α. The isoelectric point of his globulin α at 0° was pH 5.06.

The lower curves in Fig. 1 are our data for the mobility of albumins A and B at a concentration of 0.1 per cent. These data are not directly comparable with those of Kekwick because of the difference in ionic strength (held at 0.1 instead of 0.02 to keep the globulins in solution when present) but they do show that there is no significant difference in the behavior of the two proteins over the range he investigated, pH 4.2 to 5.5. A few points

measured at $\mu = 0.02$ showed that our present preparations agreed completely with those previously reported (2, 6, 8, 12, 17). Below pH 4.2 and above pH 5.5 (Fig. 1), the differences in mobility are quite definite.[4] This was shown by both of our sets of preparations. In addition, data of Moyer and Abramson (17) for a preparation of serum albumin isolated by Moyer (8) are plotted for comparison. No attempt had been made to isolate the

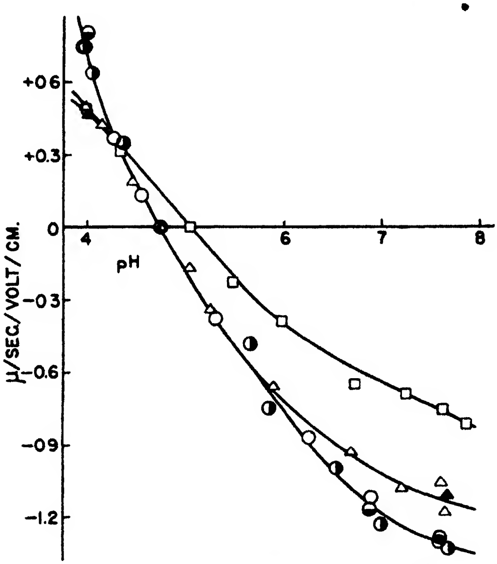

FIG. 1. The electric mobility of horse serum proteins adsorbed on quartz or collodion at an ionic strength of 0.1. △▲, preparations of Kekwick's albumin A; ○◐⊖, preparations of his albumin B; □, total globulin. The albumins were at 0.1 per cent and the globulin at 0.3 per cent. Smooth curves have been drawn by eye to fit the points.

constituents; yet the curve agrees completely with the present data for albumin B. Moyer used Na_2SO_4 as a crystallizing salt, followed by extraction of the initial batch of crystals with water at refrigerator temperature before the refinements introduced by

[4] Luetscher (19) has recently reported that the two horse serum albumins isolated by the method of McMeekin show a difference in electric mobility at pH 4.0 but not at pH 7.4. Human serum albumin which had been crystallized also showed two boundaries of different mobility in the Tiselius instrument at pH 4.

Kekwick had been published. An essential part of Kekwick's method of separating albumins A and B is the extraction of the crystals at 2°, at which temperature albumin B is more soluble. It is therefore probable that Moyer had unintentionally isolated albumin B.

Albumin B has a definitely higher mobility than albumin A at either end of the range. Comparison of our results with those of Tiselius (3) indicates that the single, yellow serum albumin, which he isolated electrophoretically and which occurs in the serum, agrees closely with our data for albumin A, the component with the yellow color and higher carbohydrate content. In agreement with Tiselius, we find that no appreciable difference is produced in these results by a change in the ionic species of the buffer system. The data below pH 5.8 were secured in acetate buffers and above this value in phosphates at the same ionic strength. Of course, a shift in the total ionic strength will affect both the mobility and the isoelectric point (2, 6, 7). The isoelectric point of all our preparations of albumins A and B had the same value, pH 4.75, at this ionic strength. Tiselius (3) reports a value of pH 4.64 for albumin in solutions of the same ionic strength but at 0°.

Quartz and collodion particles and mineral oil droplets were next placed in whole serum and, after a sufficient time had elapsed for coating, these serum suspensions were then diluted to 1:50. In Fig. 2 is shown the electrophoretic behavior of these particles. It is clear that the electric mobility of quartz is quite markedly higher than mineral oil or collodion, which follow the same curve. Evidently these latter particles have adsorbed a different constituent than that taken up by the quartz. No differences could be found between the behavior of serum samples from different sources. The smooth curves in Fig. 2 are taken from the data in Fig. 1 for the electric mobility of the separated fractions. The curve in Fig. 2 which fits the data for quartz particles is that of albumin A. It will be noticed that the fit is quite good, except for a slight divergence on the acid side of the isoelectric point, indicating that quartz placed in serum adsorbs a film of albumin A. Tiselius has found that the electric mobilities of the serum proteins in the isolated state are slightly different from their mobilities when in serum, so that the shift may possibly be ascribable to this. The upper smooth curve which fits the data for

mineral oil and collodion is the smooth curve from Fig. 1 for globulin. It thus seems that the hydrophilic quartz adsorbs the more hydrophilic serum albumin, while the hydrophobic mineral oil and collodion surfaces become coated with the relatively hydrophobic globulin. This is corroborative evidence that serum consists of definite constituents which correspond in their behavior to components isolated by chemical treatment. Although some experiments with other types of particles[5] were attempted, no trace

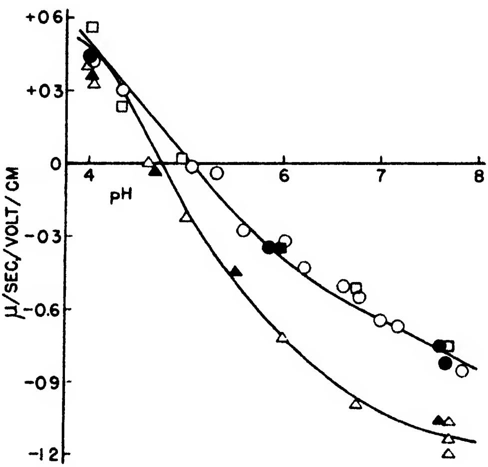

FIG. 2. The electric mobility of quartz (△▲) and collodion (○●) particles and mineral oil (□ ■) droplets in horse serum at a dilution of 1:50 and an ionic strength of 0.1. The closed and open symbols represent the behavior of horse sera from different sources. The smooth curves are the same as those in Fig. 1 for globulin and albumin A. The agreement with present data indicates that the particles have adsorbed definite constituents from the serum.

of albumin B was ever found by this means. It is possible that albumin B with its low carbohydrate and bilirubin content is to some extent a laboratory product.

Dialysis of serum against 1 per cent NaCl had no apparent in-

[5] Experiments with glass (Pyrex) particles showed that these adsorb a component different from the other particles investigated. Although the curve of glass followed that for quartz down to pH 6.0, below this point it diverged, becoming isoelectric at pH 4.3. The nature of its surface is problematical.

fluence on the results. In agreement with Abramson (11), the serum concentration did not influence the mobility when enough was present to coat, up to amounts which began to affect the ionic strength of the buffer. That these two particles, quartz and collodion, actually adsorbed different components was shown by coating each with serum and mixing the two suspensions, whereupon the frequency histogram of mobilities showed two modes.

Rabbit Serum

Fig. 3 shows the behavior of quartz and collodion particles when suspended in 1:50 dilutions of rabbit serum. It will be

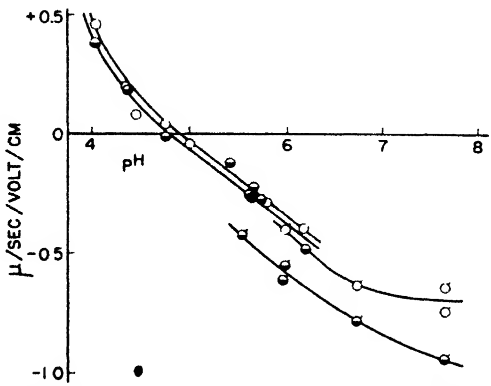

Fig. 3. The electric mobility of quartz (⊖) and collodion (○) particles in rabbit serum at a dilution of 1:50. The tagged circles represent data obtained in phosphate buffers; the remaining data were measured in acetate buffers at the same ionic strength, 0.1. The smooth curves have been drawn by eye to fit the points.

seen that there is no significant difference between the mobilities of these two types of particles between pH 4 and 6.2. There was a slight shift in the pH of the isoelectric point but this was difficult to measure accurately. The isoelectric point lay between pH 4.8 and 4.9 for both surfaces. If the buffer was changed from acetate to phosphate, the same ionic strength being retained, the results in phosphate were quite clearly different for the two particle types and the curves were shifted in position so that a break came between the acetate and phosphate data without much change in slope. The quartz surface is much more markedly affected than the collodion by the change in buffer.

The results indicate that the coated quartz and collodion particles in acetate buffer really have different surface films, although their mobilities are quite similar. When such particles are placed in phosphate buffers, the coating of the quartz seems to adsorb phosphates to a greater extent than that on the collodion. These data emphasize that similarity in electrophoresis does not necessarily mean identity in constitution. A few experiments with mineral oil gave the same mobility values as collodion.

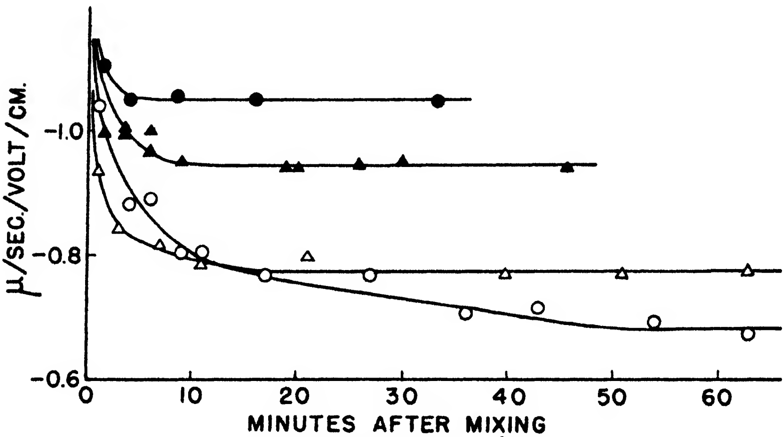

FIG. 4. Changes in the electric mobility of quartz and collodion particles caused by adsorption of protein constituents from horse and rabbit sera. The particles were added to a 1:50 dilution of serum, $\mu = 0.1$, pH 7.6. △, collodion, ▲, quartz, in horse serum; ○, collodion, ●, quartz, in rabbit serum.

The above experiments were performed after a sufficient length of time was allowed for complete coating (about 1.5 hours). To ascertain the manner in which the surfaces change immediately after exposure to horse or rabbit serum, we made 1:50 dilutions of serum at an ionic strength of 0.1 phosphate buffer, pH 7.6. Quartz or collodion particles were added and electrophoresis measurements performed at successive time intervals after mixing. Fig. 4 shows that both collodion and quartz attain uniform surfaces in horse serum in less than 10 minutes. On the other hand, these particles in rabbit serum were quite differently affected by time; the quartz became constant in about 5 minutes

but the collodion took nearly an hour to reach constancy. It seems probable that these results are influenced by differences in the rates of diffusion to the surface and the concentrations of the proteins being adsorbed, as discussed by us elsewhere (9).

Human Serum

Our data for the electric mobility of quartz and collodion particles suspended in human serum are shown in Fig. 5. Here too it is evident that the quartz and collodion are adsorbing different

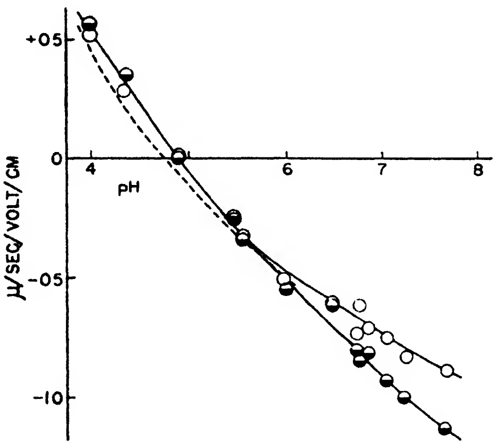

FIG. 5. The electric mobility of quartz (⊖) and collodion (○) in human serum at a dilution of 1:50, μ = 0.1. The dashed curve represents the data of Abramson for quartz and mineral oil surfaces in human serum at a slightly different ionic strength. The data above pH 6 were measured in phosphate buffers, the rest in acetate buffers.

substances but the mobility curves of these two surfaces become identical, within the limits of error, below pH 5.8 and have a common isoelectric point at pH 4.9. In common with horse serum, no break in the curve was found when the buffer system was changed from phosphate to acetate. Stenhagen (4) has reported on the electrophoresis of the protein components of diluted human plasma (at μ = 0.1) by the Tiselius method. He finds this contains fibrinogen, serum albumin, and three globulins, α, β, and γ. Of the three, globulin α is most similar in electric mobility to the films on the collodion particles, while the albumin

is closest in behavior to the coating on the quartz. Stenhagen likewise finds that the curves for these two proteins in serum merge below pH 5.5. His value for the isoelectric point of isolated albumin was pH 4.64 at 0°. He states that the concentration of globulin α is roughly 0.25 per cent in whole serum, so that if the collodion is adsorbing this constituent it is exerting a highly selective action. The results of Abramson (11) for the electrophoresis of quartz and mineral oil in 1:50 human serum dilutions are represented in Fig. 5. Our values and his are at somewhat different ionic strengths, so that one would not expect complete agreement. Our results do indicate, however, that his discovery of the identity in behavior of quartz and mineral oil surfaces under these conditions is correct. This is no proof that the two surfaces are identical, for our curves diverge definitely above pH 6.0.

Ludlum, Taft, and Nugent (20) have investigated the electrophoresis and wetting properties of the chylomicron emulsion which appears in the blood after fats have been eaten. They find these droplets to be composed of fat with an adsorbed coating which gives them an isoelectric point at pH 4.9 at an ionic strength of 0.2. They suggest that this indicates a protein surface. This value is in complete agreement with the isoelectric point which we find for quartz and collodion particles in human serum and further indicates that the droplets are coated with a protein, possibly the constituent coating the collodion, although, since mobility curves for the chylomicrons are not available, definite conclusions cannot be made. It should be mentioned that Tiselius (21) finds that the fat droplets in horse serum move with the globulin β. This is surprising in view of our finding that mineral oil droplets adsorb a constituent most similar in mobility to globulin α. Until results for adsorbed and dissolved components are available at the same temperature, this dilemma cannot be resolved.

SUMMARY

Particles of quartz and collodion adsorb different protein constituents from horse serum, as evidenced by their electrophoretic mobilities after coating. Mineral oil droplets assume surfaces identical in behavior with the collodion, within the limits of error. Comparison of these data with the electrophoresis curves for the purified globulin fraction and for the two albumins crystallized

by the method of Kekwick and adsorbed on particles showed that the quartz particles probably adsorb an albumin component agreeing with Kekwick's albumin A, while mineral oil and collodion appear to take up a globulin, possibly the globulin α of Tiselius. Although albumins A and B have identical electrophoretic mobilities, when adsorbed on particles, over the range pH 4.2 to 5.5 (investigated by Kekwick with the dissolved albumins), at either end of this range their mobilities diverge, with albumin B moving faster than A. Since no trace of albumin B was found by exposing particles to serum and since the single yellow albumin constituent isolated by Tiselius agrees most closely with albumin A, it may be possible that the colorless albumin B is to some extent a laboratory product.

These particles behaved similarly in dilute rabbit serum. Here the mobilities are affected by the ionic species of the buffer. In acetate buffers, the mobilities of the two types of particles were nearly identical, while in phosphate buffers they were definitely different. A much longer time is needed to produce a complete film on collodion surfaces in rabbit serum than in horse serum when these are introduced into initially diluted serum. Quartz particles rapidly attained a complete film in either serum.

Quartz and collodion particles in diluted human serum behaved identically, within the limits of error, between pH 4.0 and 5.8. Above this range, the two surfaces showed divergent mobilities. In comparison with the data of Stenhagen, it seems probable that the quartz becomes coated with albumin and the collodion with globulin α. The common isoelectric point of these particles agreed with that already found for the surfaces of the chylomicron emulsion in blood.

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A CRITICAL STUDY OF THE *p*-DIMETHYLAMINOBENZALDEHYDE METHOD FOR DETERMINING TRYPTOPHANE AS CONTRASTED WITH THE GLYOXYLIC ACID METHOD*

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The *p*-dimethylaminobenzaldehyde method of estimating tryptophane as modified by May and Rose (1) consisted in incubating a suspension of the protein in a hydrochloric acid solution of the aldehyde reagent at 37° for 6 to 7 days. Casein similarly treated was employed as the standard for color comparison and was assumed to contain 1.5 per cent tryptophane. Holm and Greenbank (2), using pure tryptophane as the standard, found that the bluish violet color reached maximum intensity only after 7 to 9 days incubation and that the relative intensities of the color given by tryptophane and casein indicated that casein contained 2.24 per cent tryptophane. Sullivan, Milone, and Everitt (3) have recently shortened the procedure by heating the mixture of casein and reagent at 85° for 15 minutes and adding dilute H_2O_2. The color developed by a given amount of casein in this short procedure was found to be identical, in shade and intensity, with that given by the same amount of casein in the long procedure. However, tryptophane could not be employed as the standard in the short procedure because it is unstable in hot acid solution. Casein was recommended therefore as the standard and was said to contain 2.4 per cent tryptophane; *i. e.*, the value obtained by them in the long procedure.

Recently we described a modification of the glyoxylic acid method (4) by which a freshly prepared, air-dried sample of casein was found to contain 1.19 per cent tryptophane. The trypto-

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phane content of casein as determined by the aldehyde method is therefore approximately twice that obtained by the glyoxylic acid method or by ultraviolet spectrophotometry (5). In the aldehyde method it is assumed that, under the conditions of the long procedure, free tryptophane and an equal amount of tryptophane in the casein molecule react in an identical manner with *p*-dimethylaminobenzaldehyde and give colors of equal intensity. The observations recorded in this paper show that this is an erroneous assumption and is responsible for the discrepancy in the results by the two methods.

EXPERIMENTAL

In all of the tests described herein in which the short and long procedures were used, strict adherence to the conditions recommended by Sullivan and coworkers was observed. The color measurements were made with the Evelyn photoelectric colorimeter (6), with the 600 mμ light filter. From the results obtained with varying amounts of *l*-tryptophane in the long procedure a calibration curve was prepared. The color reaction conforms approximately with Beer's law.

Numerous determinations of tryptophane were made by the aldehyde and glyoxylic acid methods on casein, casein hydrolysates, tryptophane, and tryptophane derivatives. It was found that if a tryptophane or casein solution in HCl-aldehyde mixture was incubated for 24 hours at 37° and a 5 cc. aliquot transferred to 15 cc. of water in a colorimeter tube and 3 drops of 3 per cent H_2O_2 added, the color reached its maximum in 30 to 40 minutes, remained stable for 10 to 15 minutes, and then faded. This procedure is referred to in Table I as a modified procedure. It will be seen that it gives practically the same result as the long procedure in the case of casein but free tryptophane developed more color than in the long procedure. From the results summarized in Table I the following observations may be made.

If the casein is hydrolyzed with $Ba(OH)_2$ and the long procedure applied to the hydrolysate, after the $Ba(OH)_2$ is removed with H_2SO_4, the result is of the same order as with the glyoxylic acid method but only about 50 per cent of the value obtained by the long procedure. The addition of hydrogen peroxide, in the modified procedure, intensifies the color. The glyoxylic acid

method gives the same result when applied to (*a*) a solution of casein in 5, 10, or 20 per cent NaOH or in 5 per cent HCOOH and (*b*) a $Ba(OH)_2$ hydrolysate of the protein with or without subjection to mercuric sulfate precipitation (4). Tryptophane derivatives give more color in the long procedure than an equivalent amount of tryptophane and the color is enhanced by H_2O_2 in the modified procedure. This is not the case with the glyoxylic acid method in which only hypaphorine behaves abnormally.

TABLE I

Comparison of Tryptophane Determinations by p-Dimethylaminobenzaldehyde Method and Glyoxylic Acid Method

The results for the tryptophane derivatives are expressed on the basis of an amount of each substance equivalent to 1.0 mg. of tryptophane.

Substance tested	*p*-Dimethylaminobenzaldehyde method		Glyoxylic acid method
	Long procedure	Modified procedure	
	mg.	*mg.*	*mg.*
Casein, 100 mg. (air-dried)	2.4	2.5	1.2
" 100 " (baryta hydrolysate)	1.3	1.9	1.2
Edestin	2.6		1.3
Tryptophane	1.0	1.5	1.0
Abrine	1.4	2.0	1.0
Hypaphorine	Red color	2.0	0.6
Tryptophylglycine	1.3	2.0	1.0
Glycyltryptophane	1 4	2.0	1.0
Tryptophane methyl ester hydrochloride	1.4	2.0	1.0
Acetyltryptophane	1.4	2 0	1.0

These results indicate that two factors are involved in the color reaction of the *p*-dimethylaminobenzaldehyde method; namely, the mode of linkage of the tryptophane and the degree of oxidation. The existence of the first factor is confirmed by the difference in the behavior of casein and casein hydrolysates and by the different behavior of the tryptophane derivatives compared to free tryptophane. Considering the second factor, it is apparent that unhydrolyzed casein is able to cause the color reaction to go to completion in the long procedure without the addition of an oxidizing agent, some as yet unidentified constituent of casein having the effect of an oxidizing agent. In the short procedure

of Sullivan *et al.*, no increase in color resulted from the addition, to casein, of each of the amino acids which it is known to contain. Zein, proline, oxyproline, gelatin, or an acid hydrolysate of casein, when added to tryptophane in the long procedure, had no effect on the final color intensity. The addition to casein of galactose or the phosphopeptone of casein (7) in the short and long procedures had no effect. When calcium phosphate or phosphoric acid was added to casein in the long procedure, the color developed more rapidly but the final intensity of color was not changed. While many investigators have assumed that the mechanism of the color reaction between tryptophane and an aldehyde is the same for all aldehydes, the results in Table I indicate that tryptophane reacts differently with glyoxylic acid and *p*-dimethylaminobenzaldehyde. All indole compounds react with the aldehyde reagent in the modified procedure to give the same color, as confirmed by the absorption spectrum. In addition to the compounds listed in Table I, indole, skatole, indoleacetic acid, indolepropionic acid, and indolebutyric acid were tested. The glyoxylic acid method gives different colors with these indole compounds but the same color with the tryptophane derivatives with the exception of hypaphorine.

Hopkins and Cole (8), Dakin (9), and Onslow (10) have reported the isolation from casein of 1.5 to 1.7 per cent of tryptophane and these figures are often quoted in support of colorimetric methods which give values greater than this. Lüscher (11), while working with Hopkins, used a colorimetric method for the estimation of tryptophane in casein and found 1.10 per cent. It is evident that Hopkins could not have regarded his own figure, obtained by isolation, as representing the true tryptophane content of casein, and further, a careful perusal of Onslow's paper fails to substantiate his claim to have confirmed Dakin's finding but indicates, rather, that the yield was 1.1 to 1.5 per cent. The isolation of tryptophane from casein by Onslow's modification of Dakin's method has therefore been reinvestigated by us. The only change in the procedure was to introduce a second mercuric sulfate precipitation after the second digestion and before extraction with butyl alcohol. All filtrates and residues, which are usually discarded in the procedure, were worked up and examined for tryptophane by the glyoxylic acid method. The purity of the trypto-

phane isolated was established by colorimetric analysis and by direct and mixed melting point determinations. The results of two experiments are given in Table II. We have been unable to obtain evidence by isolation for the presence in casein of more tryptophane than is indicated by the direct application of the glyoxylic acid method.

TABLE II

Isolation of Tryptophane from Casein

	200 gm. casein		160 gm. casein	
	gm.	*per cent*	*gm.*	*per cent*
By colorimetry	2.20	100.0	1.76	100.0
Actually isolated (by weight)	1.43	65.0	1 25	71.0
Lost during concentration, extraction, and recrystallization (by colorimetry)	0.37	16.8	0.25	13.9
Lost in discarded filtrates and residues (by colorimetry)	0 18	8.3	0.14	7.7
Total accounted for	1.98	90.1	1.64	92.6

SUMMARY

The *p*-dimethylaminobenzaldehyde method for the estimation of tryptophane in proteins is shown to give erroneous results owing to the fact that tryptophane as combined in the protein molecule gives more color with the aldehyde reagent than does an equivalent amount of free tryptophane. The mode of linkage and degree of oxidation of tryptophane influence the color reaction, so that the source of error in the procedure is the use of free tryptophane as the standard. The reliability of the glyoxylic acid method is confirmed.

Observations are recorded which indicate that current theories on the mechanism of the reaction between aldehydes and tryptophane require revision.

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THE EFFECT OF INSULIN UPON UREA FORMATION, CARBOHYDRATE SYNTHESIS, AND RESPIRATION OF LIVER OF NORMAL AND DIABETIC ANIMALS*

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The severely diabetic subject has an excessive protein metabolism which returns to normal when sufficient carbohydrate utilization is reestablished. The current hypothesis to explain this is that restoration of the ability to oxidize carbohydrate spares protein, and the metabolism returns to normal.

An alternative hypothesis, namely that insulin has a direct specific effect on protein metabolism, is also a possibility. Recently, Bach and Holmes (1937) found evidence for it on the basis of experiments with slices of normal fasted rat liver equilibrated in bicarbonate buffer. They found, both with no added substrate and with *dl*-alanine, that insulin, when added to the medium, partially inhibited urea formation and carbohydrate synthesis. They concluded that a function of insulin is the suppression of glyconeogenesis by the inhibition of oxidative deamination of glycogenic amino acids.

The importance of this hypothesis concerning a specific action of insulin upon protein metabolism led us to test it extensively. Our experiments indicate that a possible rôle of insulin in protein metabolism is the regulation of the deamination by the liver of the *d* isomers (so called unnatural isomers) of glycogenic and non-glycogenic amino acids.

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Methods

Our experiments were performed entirely with liver slices. Rats were killed by decapitation, cats by concussion. No anesthetic was used. The liver slices were cut with the double cutter of Thomas and De Eds (1937) and were 0.40 ± 0.05 mm. thick. As a rule the slices (100 to 200 mg.) were placed in the equilibrating (conical Warburg) vessel without washing. Speed in preparation of the slices was emphasized and in most of the experiments the time from killing to the beginning of the equilibration in 100 per cent oxygen at 38° was 20 to 30 minutes.

The medium was 3 cc. of Krebs' phosphate saline solution at pH of 6.8 or 7.4. Amino acids were added where indicated. Equilibration was carried out for 2.0 hours.

The oxygen uptake is expressed in micromoles of O_2 per gm. of wet liver.

Urea + NH_3—These were determined together on the samples of tissue + medium after deproteinization with trichloroacetic acid by a method similar in principle to that of Van Slyke and Cullen (1914). Following nesslerization of the ammonia solution, readings were made with the Evelyn photoelectric colorimeter. Frequent controls assured us that amounts of urea down to 0.3 micromole could be recovered with an accuracy of ±2 per cent. NH_3 + urea is expressed together in terms of micromoles per gm. of "urea."

Insulin[1]—The insulin used was either Stearns' or Lilly's crystals of zinc insulin or Lilly's zinc-free insulin powder assayed at 22 to 23 clinical units per mg.

Fermentable Carbohydrate—After hydrolysis at 100° in 1 N H_2SO_4 for 2 to 3 hours, both tissue and medium were deproteinized with tungstic acid. 2 aliquots of the centrifugate were analyzed for total reducing substances by the method of Shaffer, Hartmann, and Somogyi (*cf.* Peters and Van Slyke, 1932). A third aliquot was fermented with washed yeast and the residual non-sugar reducing substances determined. The fermentable carbohydrate was calculated from the difference. Results are expressed in glu-

[1] We are indebted to Dr. F. B. Peck of the Lilly Research Laboratories and to Dr. Melville Sahyun of the Frederick Stearns and Company Scientific Laboratories for generous supplies of these purified insulin preparations.

cose equivalents as mg. of total fermentable carbohydrate per gm. of wet liver.

Animals Used—These included normal rats fasted 24 hours, non-fasted rats with demedullated adrenals, normal cats fasted 24 to 48 hours, depancreatized (diabetic) cats (48 hours after operation), hypophysectomized-depancreatized (Houssay) cats, and cats injected with alkaline extracts of the anterior pituitary. The completeness of the pancreatectomy was confirmed by autopsy and by the degree of glycosuria.

Amino Acids—All preparations were those of Hoffmann-La Roche.

Duplicate Samples—As a rule, duplicate samples of liver slices were set up in each type of medium. In this way, the reproducibility of the oxygen uptake, urea formation, and carbohydrate synthesis could be determined.

Statistical Presentation of Data—Whenever possible, our results are given in statistical form. The number of animals and the number of samples of liver in each category of experiment are given. The arithemetical mean together with the standard error of the mean is given. The standard error of the mean was calculated by the short method of Peters (*cf.* Mellor (1909)); *viz.*,

$$\text{Standard error or mean} = \frac{1.25\ \Sigma \text{ deviations from mean}}{n\sqrt{n-1}}$$

Terminology—This is the same as that used by Bach and Holmes, and for convenience the terms are tabulated here.

Basic observations	
1. Initial	Value from slices killed immediately
2. Final plain	Value after equilibration for 2 hrs.; no substrate
3. " with amino acid	Value after equilibration; amino acid present
4. " plain with insulin	Value after equilibration with insulin; no substrate
5. " with amino acid and insulin	Value after equilibration with insulin; amino acid present
Effects calculated	
6. Increase plain	(2) − (1)
7. Amino acid effect	(3) − (2)
8. Insulin effect plain	(4) − (2)
9. " " with amino acid	(5) − (3)

Effect of Insulin upon Deamination by Liver Slices from Normal Fasted Rat

Our results are shown in Table I. The effects of insulin were dependent upon the type of substrate. With no added substrate we found an increase rather than a decrease of urea formation. With *dl*-alanine we regularly found a decrease which appears significant. We also studied deamination of the optical isomers of alanine. We found in the case of *l*(+)-alanine (the natural isomer) no significant effect of insulin. With *d*(−)-alanine, however, we found in more than half of the rats a significant inhibition of deamination.

TABLE I*

Normal Rat Liver Slices; Urea Formation and Effect of Insulin Thereon

	No. of rats	No. of samples	Urea mean
			micromoles per gm. per 2 hrs.
Initial	30	44	9.5 ± 0.37
Increase plain	24	36	7.5 ± 0.60
Amino acid effects			
dl-Alanine (0.020 M)	6	6	25.1 ± 3.30
l(+)-Alanine (0.020 M)	7	8	5.7 ± 0.60
d(−)-Alanine (0.020 ")	6	11	29.0 ± 4.00
Insulin effects (1 unit per cc.)			
Plain	9	17	+1.0 ± 0.22
	1	1	−1.0
With *dl*-alanine	6	8	−5.0 ± 1.00
" *l*(+)-alanine	3	9	+2.4 ± 1.30
	3	10	−2.7 ± 2.40
" *d*(−)-alanine	3	8	+1.9 ± 0.53
	5	8	−4.5 ± 1.30

* In this and subsequent tables, the mean amino acid and insulin effects are reported in two groups; *viz.*, those giving increases (+) and those giving decreases (−). Insulin concentrations in this and the following tables are expressed in clinical units per cc.

Deamination Following Insulin Injection—It is possible that the diffusion of insulin from the medium into the liver cells is slow or does not occur at all. We therefore sought to enhance the action of insulin by prior intravenous injection of insulin into the intact

animal, followed 1 to 1.5 hours later by a study of deamination by the liver slices. Our results are shown in Table II.

For normal rats we found that neither the "increase plain" nor the "$l(+)$-alanine effect" was significantly different from those of the control group. With $d(-)$-alanine, however, there is considerably less urea formation, and this finding is in conformity with the previous demonstration that insulin partially inhibits the deamination of $d(-)$-alanine only.

Table II

Effect of Injection of Insulin into Intact Rats upon Subsequent Rate of Urea Formation by Liver Slices

1 to 3 units of insulin was injected 1 to 3 hours prior to the experiment; the results are expressed in micromoles per gm. of urea per 2 hours.

	Injected normal rats			Uninjected controls	Difference
	No. of rats	No. of samples	Mean urea	Mean urea	
Increase plain	5	6	8.4 ± 0.9	7.5 ± 0.6	+0.9 ± 1.1
$l(+)$-Alanine	4	6	7.0 ± 1.4	5.7 ± 0.6	+1.3 ± 1.5
$d(-)$-Alanine	4	6	18.5 ± 8.5	29.0 ± 4.0	−10.5 ± 9.2
	Adreno-demedullated rats (injected)				
Increase plain	4	8	9.8 ± 1.1	10.3 ± 2.1	−0.5 ± 2.4
$l(+)$-Alanine	2	2	7.7 ± 2.8	7.1 ± 2.5	+0.6 ± 3.8
$d(-)$-Alanine	4	4	13.9 ± 3.3	15.6 ± 0.4	−1.7 ± 3.3

Deamination in Adreno-Demedullated Rats—There is a possibility that adrenalin might have a "contrainsular" action. Since the traumatic death of the rats might considerably enhance this, we sought to eliminate it by using adreno-demedullated rats. These rats had been operated on 6 weeks prior to use. We found in these animals, however, that the liver slices obtained 1 to 1.5 hours after intravenous insulin showed an "increase plain" and a "$l(+)$-alanine effect" quite similar to those of the normal group, indicating again that insulin has no effect upon these two types of deamination. With $d(-)$-alanine we found deamination low in the injected group, but comparison with an uninjected control group, in other respects treated similarly, also showed a very low

"$d(-)$-alanine effect"; *i.e.*, we failed to demonstrate any inhibition in this type of preparation.

Deamination of $l(+)$-Alanine with Higher Concentrations of Insulin—Even when the concentration of insulin was increased beyond the usual 1 unit per cc., no effect could be shown upon the deamination of $l(+)$-alanine. In Table III we give the increase of urea without insulin and the ratios of urea formation with insulin to this basic value. In the mean the ratio is 1.00 ± 0.05; *i.e.*,

TABLE III

Normal Rat Liver Slices; Effect of Zinc Insulin at Varying Concentrations upon Deamination of $l(+)$-Alanine (0.030 M) at 38° in 2 Hours

Rat No.	Insulin	Basic increase of urea; no insulin	Ratio of increase of urea with insulin to increase without insulin	Rat No.	Insulin	Basic increase of urea; no insulin	Ratio of increase of urea with insulin to increase without insulin
	units per cc.	*micromoles per gm.*			*units per cc.*	*micromoles per gm.*	
9C	0	27		9E	0	21	
	5.5		0.89		5.5		0.99
	11		0.87		11		1.08
	17		0.67		16.5		0.90
	22		1.05		22		1.05
9D	0	18		9F	0	24	
	5		1.34		7.5		1.01
	10		1.18		11		0.88
	15		1.24		17		0.93
	20		1.37		22		1.03
Mean of all ratios (4 rats, 16 samples) =							1.00 ±0.05

the data show no consistent effects of insulin from 1 to 22 units per cc.

Deamination in the Presence of Arsenite—Arsenite inhibits the major portion of the oxygen uptake of liver slices, but not that due to oxidative deamination (Krebs, 1933). With arsenite in the medium, we were able to show that insulin inhibited by 10 to 20 per cent the deamination of $d(-)$-alanine, but had no effect upon the deamination of $l(+)$-alanine (Table IV). This is in conformity with

our previous data which show that inhibition by insulin of deamination, when it does occur, is on the unnatural *d*(−)-alanine rather than on *l*(+)-alanine. The inhibition of oxygen uptake found with arsenite was correlated with the effect upon deamination. In the case of *l*(+)-alanine, there was no significant effect upon the oxygen, whereas with *d*(−)-alanine there was found an inhibition which in ratio to the inhibition on urea formation was 1.0 ± 0.6, essentially that expected theoretically.

Action of Insulin upon Deamination of Non-Glycogenic Amino Acids—The possibility that insulin might inhibit the deamination of non-glycogenic amino acids was tested. In the case of valine

TABLE IV

Effect of Insulin (1 Unit per Cc.) upon Urea Formation by Liver Slices from the Normal Rat

Phosphate buffer + 0.001 M arsenite.

Rat No.	No. of slices	Insulin effect with 0.020 M *d*(−)-alanine. Urea formation (2 hrs.)	Rat No.	No. of slices	Insulin effect with 0.020 M *l*(+)-alanine. Urea formation (2 hrs.)
		micromoles per gm.			*micromoles per gm.*
5E	1	−7.5	5B	2	−0.4
5F	1	−6.1	5D	2	−1.4
5G	1	−4.8	5E	1	−0.3
5H	1	0.0	5G	1	0.0
38	1	+3.8	5H	1	−0.3
39	4	−5 4	38	1	−0.3
Mean	9	−4.0 ± 1.2		8	−0.4 ± 0.2

and leucine (Table V) there was found no inhibition of the natural *l* isomers, even at high concentrations of insulin. With the unnatural *d* isomers, we found, as with *d*(−)-alanine, significant inhibition in some cases. With another non-glycogenic amino acid, *dl*-methionine, we regularly found inhibitions, sometimes as high as 50 per cent. The optical isomers of this acid were not available to us.

Urea Formation by Liver Slices of Normal and Diabetic Cats—The data in Table VI show in the diabetic cat an "increase plain" 46 per cent greater than in the normal. It appears that the increased protein metabolism in the diabetic is associated with

hyperactivity of one specific enzyme system; namely, the oxidative deaminase system of the liver. There is also a slightly increased deamination of *dl*-alanine (10 per cent).

The effect of insulin in the normal and diabetic cat was essentially that found in the normal rat; *viz.*, no effect of insulin upon the "increase plain," and an inhibitory effect with *dl*-alanine.

TABLE V

Normal Rat Liver Slices; Effect of Insulin upon Urea Formation in 2 Hours in Presence of Non-Glycogenic Amino Acids; 38°

Rat No.	Increase with no insulin. Urea formation	Insulin	Insulin effect. Urea formation	Rat No.	Increase with no insulin. Urea formation	Insulin	Insulin effect. Urea formation
	With 0.020 M *l*(+)-valine				With 0.020 M *d*(−)-valine		
	micromoles per gm.	*units per cc.*	*micromoles per gm.*		*micromoles per gm.*	*units per cc.*	*micromoles per gm.*
7	11.1	1	−0.5	8	23.6	1	+1.0
		5	+0.4			5	+2.4
		10	−0.4			10	−1.3
		20	−0.4			20	−3.7
	With 0.020 M *l*(−)-leucine				With 0.020 M *d*(−)-valine		
9	11.0	1	+0.6	34	16.6	10	+3.1
		5	+0.4		With 0.020 M *d*(+)-leucine		
		10	−0.4				
		20	−0.6	9	24.6	5	−2.5
	With 0.020 M *dl*-methionine					10	−2.8
						20	−5.6
37A	49.0	1	−21.2		With 0.020 M *d*(+)-leucine		
37B	11.1	1	−1.7				
37C	14.1	1	−6.3	34	25.4	10	+1.4
37D	13.6	1	−4.0				
37E	10.5	1	−5.5				

One might expect that the inhibitory effect of insulin would be greater in the diabetic cat, but our experiments did not show this to be the case (Table VI).

Urea Formation in Houssay Cats—Insulin failed to restore to normal the excessive deamination of depancreatized cat liver slices. In contrast were our findings with two hypophysectomized-depancreatized cats. Here (Table VII) we found an

"increase plain" essentially that of the normal cats and a "*dl*-alanine effect" less than normal. Insulin effects in these animals were not significant.

Increased Urea Formation by Liver Slices in Diabetic Cats Receiving Prior Injections of Anterior Pituitary—In four of five completely or partially depancreatized cats receiving for 4 to 10 days

TABLE VI

Normal and Diabetic Cat Liver Slices; Urea Formation Plain and with dl-Alanine and Effect of Insulin Thereon at 38° in 2 Hours

	Normal			Diabetic			Difference
	No. of cats	No. of samples	Mean urea	No. of cats	No. of samples	Mean urea	
			micromoles per gm.			*micromoles per gm.*	*micromoles per gm.*
Initial	7	21	11.9 ± 0.7	9	26	16 3 ± 0 3	+4 4 ± 0.7
Increase plain	7	17	11.2 ± 1.0	9	25	16.4 ± 2.5	+5 2 ± 2.7
dl-Alanine	7	13	37.4 ± 2 9	5	10	41.4 ± 6.5	+4.0 ± 7.2
Insulin effects (1 unit per cc.)							
Plain (no substrate)	1	1	+1.2	4	7	+2.7	
	2	5	−1.4 ± 0.3	4	3	−3.1	
Mean	3	6	−0.9 ± 0.6	4	10	+0.9 ± 1.1	
With *dl*-alanine (0.020 M)	1	1	+4.8	2	2	+11.4	
	2	5	−5.5 ± 1.8	9	9	−6.1 ± 0.7	
Mean	3	6	−4.9 ± 2.1	9	11	−2.9 ± 1.1	

prior to the experiments daily injections of anterior pituitary extract, we found (Table VIII) values for urea formation significantly higher than normal. It is to be noted, too, that here our greatest insulin effects were observed. These positive results, together with our experiments on the Houssay cat, are in accord with the hypothesis that the anterior pituitary has a "contrainsular" activity.

Effect of Insulin upon Deamination of d(−)-Alanine by Kidney

Slices—The rate of deamination of $d(-)$-alanine by slices of kidney cortex is much greater (300 ± 100 microequivalents of NH_3 per gm. per 2.0 hours) than by liver slices. Our results (Table IX)

TABLE VII

Urea Formation and Influence of Zinc Insulin Thereon; Liver Slices from Two Houssay Cats with No Added Substrate and with dl-Alanine at 38° in 2 Hours

Five samples in each experiment.

	Mean urea	Difference from normals (7 cats). Mean urea
	micromoles per gm.	*micromoles per gm.*
Increase plain	11.0 ± 0.9	−0.9 ± 1.1
dl-Alanine effect	22.8 ± 3.9	−10.6 ± 5.5
Insulin effects (1 unit per cc.)		
Plain	−1.0 ± 1.7	
With *dl*-Alanine	+2.2 ± 2.2	

TABLE VIII

Effect in Cats of Combining Pancreatectomy and Injections of Anterior Pituitary Extracts upon Subsequent Rate of Urea Formation by Liver Slices, and Effect of Insulin Thereon

Cat No.	Order of operations	Urea formation, micromoles per gm. per 2 hrs., at 38°						
		In-crease plain	Amino acid effect			Insulin effect		
			dl-Alanine	*l*(+)-Alanine	*d*(−)-Alanine	*dl*-Alanine	*l*(+)-Alanine	*d*(−)-Alanine
203A	A. P., C. P.	45*	135*			−33*		
203B	" "	12.1	13.1	43.8*	29.5		−13.4*	−2.9
203C	" "	9.2	24.9	12.0*	23.8		−1.0	−2.3
10-39	P. P., A. P.	11.4		5.6			+2.7	−1.6
13-39	" "	10.8		18.7*			−10.7*	−5.8

A. P., C. P. = injections of anterior pituitary extract for 1 week followed by complete pancreatectomy. P. P., A. P. = partial pancreatectomy (no glycosuria) followed by anterior pituitary extract (with glycosuria).

* These results are significantly different from mean normal values.

in four out of five rats show an inhibition of about 12 per cent in the presence of insulin at 1 unit per cc., and are thus in essential agreement with our results with liver.

Effect of Insulin upon Oxidative Deaminase Preparation—The possibility that the inhibiting action of insulin upon the deamination of $d(-)$-alanine observed with intact cells might be due to a combination with or blocking of d-deaminase was tested with purified d-deaminase. With two preparations of cat liver deaminase and one of cat kidney deaminase prepared by the Bernheim and Bernheim (1935) method we could find no influence of insulin at 1, 2, 5, and 10 units per cc. upon the deamination of $d(-)$-alanine. Therefore, a blocking of the deaminase by insulin seems to us to be quite unlikely.

Synthesis of Total Fermentable Carbohydrate by Liver Slices of Normal Rats—We studied the effects of insulin upon the rate of

TABLE IX

Synthesis of Fermentable Carbohydrate by Liver Slices of Normal Fasted Rat and Influence of Zinc Insulin Thereon at 38° in 2 Hours

No added substrate.

	No. of rats	No. of samples	Fermentable carbohydrate
			mg. per gm.
Initial	5	9	2.95 ± 0.39
Increase plain	5	10	0.91 ± 0.20
Insulin effect (1 unit per cc.)			
Plain	5	5	+0.54
	4	4	−0.40
Mean (insulin effect)	9	9	+0.12 ± 0.20

new formation of carbohydrate from non-carbohydrate sources by liver slices of the normal rat and normal and diabetic cat. In the normal rat, following equilibration of the slices for 2.0 hours in a phosphate buffer, we were able to show a new formation of total fermentable carbohydrate. With insulin the values fluctuate above and below the quite small "increase plain" but in the mean the insulin effect is not statistically significant.

Synthesis of Fermentable Carbohydrate by Liver Slices of Normal and Diabetic Cats—Our data, shown in Table X, give some interesting contrasts between the normal and the diabetic. The normal cat liver slice shows considerable ability to synthesize new carbohydrate in the absence of added substrate (5.5 ± 2.1 mg. per

gm. per 2 hours). In the presence of *dl*-alanine (0.020 M), there was an added synthesis of new carbohydrate (4.6 ± 1.7 mg. per gm. per 2 hours) which in the mean is 137 ± 51 per cent of that expected from "*dl*-alanine effect" on urea formation on the assumption that all of the *dl*-alanine deaminated is converted into carbohydrate. In the diabetic cat, however, the "increase plain" had a mean value of 1.8 ± 0.6 mg. per gm. per 2 hours, and there was found essentially no new formation of carbohydrate from *dl*-alanine. In the face of an increased deamination the diabetic cat liver slice appears to be unable to convert the residual carbon skeleton into carbohydrate.

TABLE X

Synthesis of Total Fermentable Carbohydrate by Liver Slices of Normal Fasted Cat and Diabetic Cat (48 Hours after Pancreatectomy), and Influence of Insulin Thereon at 38° in 2 Hours

	No. of cats	No. of samples	Fermentable carbohydrate	
Normal cats				
			mg. per gm.	
Initial	7	22	15.5 ± 1.0	
Increase plain	2	2	0.0	
	4	8	+10.2 ± 2.2	
	3	4	−1.3 ± 0.4	
Mean	7	14	+5.5 ± 2.1	
dl-Alanine effect	5	10	+6.6 ± 1.6	
	1	2	−2.8	
Mean	6	12	+4.6 ± 1.7	
Insulin effects (1 unit per cc.)				
Plain	1	1	0.0	
	6	13	+1.7	
	4	8	−0.9	
Mean	7	21	+0.7 ± 0.36	
With *dl*-Alanine	5	10	+2.3 ± 0.53	
	2	3	−1.2	
Mean	7	13	+1.5 ± 0.65	

TABLE X—*Concluded*

	No. of cats	No. of samples	Fermentable carbohydrate	Difference from normal
Diabetic cats				
			mg. per gm.	*mg. per gm.*
Initial	11	25	3.8 ± 0.4	−11.7 ± 1.1
Increase plain	11	22	+2.1 ± 0.5	
	3	3	−0.4	
Mean	11	25	+1.8 ± 0 6	−3.7 ± 2.2
dl-Alanine effect	3	4	+1.4	
	3	7	−1.3	
Mean	6	11	−0.3 ± 0.5	−4.3 ± 1.8
Insulin effects (1 unit per cc.)				
Plain	6	11	+1.22 ± 0.3	
	8	11	−0.89 ± 0.4	
Mean	11	22	+0 16 ± 0.28	−0 5 ± 0.45
With *dl*-alanine	3	6	+1.30	
	5	10	−0.82	
Mean	6	16	−0.02 ± 0.32	−1.5 ± 0.72

Insulin Effects in Liver Slices of Normal and Diabetic Cats—In the diabetic one might expect the most marked insulin effects on glyconeogenesis. Nevertheless, Table X shows that there was no significant insulin effect on the new formation of carbohydrate by either normal or diabetic cat liver, either with or without added *dl*-alanine.

Urea Formation and Carbohydrate Synthesis in Bicarbonate or Phosphate Buffers—Bach and Holmes used a buffer of bicarbonate equilibrated with 95 per cent O_2 and 5 per cent CO_2. We equilibrated a considerable number of parallel slices in both types of buffer, and found that neither urea nor carbohydrate formation, with or without insulin, was significantly different in the two media.

DISCUSSION

The hypothesis that insulin is concerned, by some direct mechanism rather than an indirect sparing action, with the regulation

of protein metabolism receives significant support from the experiments of Bach and Holmes, and those reported here. Bach and Holmes observed, and we have confirmed the fact, that the deamination by rat liver slices of the racemic or *dl* form of alanine is partially inhibited by the addition of insulin to the equilibrating medium. We have also found this true for normal and diabetic cat livers. In addition we find that, when insulin is completely absent from the tissues, *i.e.* in liver slices from cats 48 hours after pancreatectomy, oxidative deamination is increased about 50 per cent above the normal. Our further experiments show, however, that the inhibitory action of insulin appears to be confined to the *d* or unnatural isomers of the acids tested (alanine, valine, and leucine). In no experiments were we able to find any inhibition of deamination of the natural or *l* isomers of these acids.

In our experiments without the addition of amino acid, the deamination which occurred was not influenced by insulin. Since the amino acids arising in the normal course of liver metabolism presumably would be *d* isomers, this observation is consistent with the above.

The possibility of the difference of action of insulin upon the two isomeric forms of amino acids is made more real by the following considerations: Krebs (1935) and others have shown that *l*-deaminase and *d*-deaminase are quite different. The former cannot readily be separated from the structure of the cells, is sensitive to cyanide and octyl alcohol, and has an undetermined composition. The latter is cyanide-insensitive, can readily be separated from the cells, and has been identified by Warburg and Christian (1938) as a combination of a specific protein with alloxazine and adenylic acid.

The mechanism by which insulin inhibits the deamination of *d*(−)-alanine remains unknown, except that it can be said that a blocking of the deaminase is unlikely, since our preparations of *d*-deaminase were quite unaffected by insulin.

The possibility, suggested by Krebs and Eggleston (1938), that the effect might be explained by the assumption that there is a limited ability of the cell to activate oxygen, and that insulin preferentially promotes the oxidation by the liver of carbohydrates or other readily oxidizable substances, is not supported by our experiments designed to test it, for we found no effect of insulin upon the oxygen uptake or the respiratory quotient of liver slices.

Neither did we find with livers of fed rats with high glycogen, nor in livers of fasted rats in the presence of added *dl*-lactate, alcohol, or glucose, an enhanced effect of insulin upon the deamination of *d*(−)-alanine, despite the presence of this readily oxidizable substances. Furthermore, we found that insulin, in the presence of small amounts of fumarate, appreciably increased the oxygen uptake of liver slices; yet its effect upon the deamination of *d*(−)-alanine was not increased.

The significance of this apparent steric selectivity also remains a matter for conjecture.

Contrainsular Activity of Pituitary—The reviews of Russell (1938) and Long and White (1938) discuss the evidence to support the assumption that insulin and an anterior lobe factor are antagonistic hormones which, by some mechanism at present unknown, exert a purely regulatory action upon carbohydrate metabolism. We have found that the exaggerated deamination by the diabetic cat liver both without added substrate and with *dl*-alanine is restored to normal in the Houssay cat. Further, we have found that the completely or partially depancreatized cat may respond to the injection of anterior pituitary extracts by an increased rate of deamination by liver slices, and when it does, insulin may at times markedly inhibit this increased deamination. These experiments, then, are in support of this "antagonistic hormone" theory.

Carbohydrate Synthesis by Diabetic Liver Slices—In our experiments with diabetic liver slices we have found an increased deamination both "plain" and with *dl*-alanine. However, the new formation of carbohydrate does not parallel this. In point of fact, new formation of carbohydrate "plain" is less than in the normal and *dl*-alanine is apparently not converted to carbohydrate. These results are in sharp contrast to the active new formation of carbohydrate and the essentially complete conversion of the *dl*-alanine deaminated to carbohydrate by the normal cat liver slice. It might be emphasized that our results are not in accord with the hypothesis that in diabetes the liver is overproducing carbohydrate from non-carbohydrate sources.

Tentative Hypothesis—In some respects our experimental results differ from those of Bach and Holmes: (1) In rats and normal and diabetic cats, we found no significant effect of insulin upon the

new formation of carbohydrate by liver slices. (2) We found that the deamination by liver slices occurring in the absence of added amino acid was not significantly influenced by insulin. (3) The inhibitory action of insulin appears to be manifest with non-glycogenic as well as glycogenic amino acids. It is hoped that further experiments will reconcile these differences, but in view of them it appears to us, at present, that the hypothesis of Bach and Holmes is too broad. We are inclined tentatively to propose a more limited working hypothesis; namely, that a possible action of insulin is to regulate protein metabolism by partially inhibiting the oxidative deamination of the *d* isomers of non-glycogenic and glycogenic amino acids. The more definitive significance of this hypothesis in the problem of the chemical action of insulin in the normal and diabetic organism is a matter for future experimentation.

We wish to express our thanks to Mildred S. Wright for her helpful criticisms and assistance in the experiments.

SUMMARY

1. The possibility that insulin may directly influence the metabolism of protein was indicated by finding that it partially inhibited the deamination by isolated liver slices of normal animals of the (unnatural) *d* isomers of amino acids. The corresponding natural or *l* isomers were unaffected. Furthermore, in the complete absence of insulin (cats 48 hours after pancreatectomy) deamination by liver slices was found to be much greater than normal.

2. The new formation of carbohydrate by liver slices of normal or diabetic animals was found to be uninfluenced by the addition of insulin to the equilibrating medium. In the diabetic cat, despite the high rate of deamination, there was found a subnormal new formation of carbohydrate.

3. The "contrainsular" activity of the pituitary was manifested in two ways. (*a*) In the hypophysectomized-depancreatized cats the hypernormal deamination by liver slices found in the depancreatized cat was restored to normal. (*b*) Diabetic cats receiving prior injections of anterior pituitary extracts showed in four-fifths of the cases hypernormal rates of liver deamination.

4. The activity of preparations of *d*-deaminase was uninfluenced by insulin.

5. The relation of these findings to the problem of the action of insulin upon intermediary protein and carbohydrate metabolism is discussed.

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THE EFFECT OF INSULIN UPON OXIDATIONS OF ISOLATED MINCED MUSCLE TISSUE*

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Recently Krebs and his coworkers (1937, 1938) proposed a cyclical mechanism by which the oxidation of carbohydrates in the tissues is catalyzed. Our understanding of this hypothesis is as follows:

Some split-product of carbohydrate, a triose or similar compound, condenses with oxalacetic acid present in the tissues to form citric acid. This citric acid formation initiates a cycle involving the successive formation of α-ketoglutarate, succinate, fumarate, malate, and finally oxalacetate, thus completing the cycle. In the course of the cyclical reactions there occurs a succession of oxidative decarboxylations by which the triose is oxidized to CO_2 and H_2O.

Using minced pigeon breast muscle suspended in phosphate buffer to which had been added abundant coenzyme in the form of boiled heart extract, Krebs and Eggleston (1938) found that the addition of the intermediates in the cycle, particularly citrate, increased the oxygen consumption above and beyond the amount necessary to oxidize completely the added citrate. For convenience the catalytic ratio

$$\frac{\text{Excess } O_2 \text{ with citrate}}{O_2 \text{ equivalent of citrate added}}$$

is defined which, when it is greater than 1, indicates catalysis. Our calculations, from all the data of Krebs and Eggleston on the

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complete system (phosphate, boiled heart extract, citrate, muscle mince), give a value for the ratio of 4.40 ± 1.50, indicating, as Krebs and Eggleston had concluded, a catalytic effect of citrate.

They also found that the addition of zinc-free insulin (1 unit per cc.) to this system caused a further increase of oxygen consumption, which we calculate from their data to be 55 ± 7 per cent. This insulin effect was not found in the absence of citrate. Krebs and Eggleston concluded that insulin acts catalytically to oxidize carbohydrate by catalyzing the oxidation of the intermediates in the cycle, particularly citrate.

The importance of the problem of the facultative or obligatory catalytic action of insulin upon carbohydrate oxidation by muscle led us to test these hypotheses further by a series of experiments similar to those of Krebs and Eggleston.

EXPERIMENTAL

The pigeons were, as a rule, fasted for 24 hours. They were killed by decapitation, plucked, and the breast muscles quickly dissected out and cooled on ice. The muscle was then minced in the cold room at 0° by the Latapie mincer, and the mince, in weighed amounts, was suspended in the appropriate buffer. The thoroughly stirred mince was then pipetted into conical Warburg vessels which had previously been set up with the indicated additions of citrate and insulin. The final volume was 3.0 cc., and the concentration of buffer alike in all vessels.

The center well was equipped with a filter roll and alkali and, after a 10 minute period for temperature equilibration, the taps were closed and the reading begun. The respiration was followed for a period of 200 to 400 minutes until it had practically ceased. Only the final oxygen consumptions, calculated as micromoles per gm. of wet muscle, are reported.

Two types of buffer were used, the initial pH being 6.8 in both.

	Final concentration M
I. $Na_2HPO_4 + KH_2PO_4$	0.100
II. " + "	0.050
$NaCl$	0.130
$MgCl_2$	0.005
KCl	0.007

Boiled Muscle Extract—This was prepared fresh from minced sheep heart by stirring with equal volumes of water at 100° for 10 minutes and filtering. The proportion of extract to the total volume was usually 1:3.

Insulin[1]—Lilly's amorphous insulin powder, assayed at 22 units per mg., was used. It contained essentially no zinc and was of the highest purity obtainable.

TABLE I

Effect of Citrate at Varying Concentrations upon Total Oxygen Uptake of Normal Pigeon Muscle Mince at 38°

Boiled sheep heart muscle extract present in all vessels; phosphate Buffer I or II; initial pH 6.8; 0.3 gm. of muscle mince in 3 cc.

Pigeon No.	Total O_2 uptake, no citrate	Citrate	Ratio of O_2 uptake with citrate to O_2 without citrate	Catalytic ratio
	micromoles per gm.	*mM per l.*		
36	224	2	1.46	0.86
		30	0.91	−0.10
37	130	2	1.24	0.34
		40	0.80	−0.01
43	201	2.6	1.64	0.59
		12.4	2.36	0.26
		20.8	2.59	0.18
46	215	0.4	1.00	0.00
		0.8	1.03	0.09
		1.2	1.20	0.38
		1.6	1.33	0.48
		2.0	1.39	0 44
62	198	4	1.75	0.91
98	200	10	1.41	0.14
101A	159	4	1.38	0.71
Mean			1.43 ± 0.12	

Effect of Citrate upon Respiration—In Table I we show the respiration of muscle mince. The second column gives the total *basic* oxygen uptake; *i.e.*, the oxygen uptake of the mince suspended in the phosphate buffer without the addition of citrate. The fourth

[1] We are indebted to Dr. F. B. Peck of the Lilly Research Laboratories for generous supplies of zinc-free insulin.

column gives the ratio of the oxygen uptake with citrate to the basic oxygen. In practically all cases there was found an increase (mean 43 ± 12 per cent) of oxygen uptake, except with high citrate, when a depression was usually found. From our values of the catalytic ratio, however, we were unable to find any evidence that citrate catalytically increased the oxygen uptake. In no case did we find a ratio greater than 1.0, even in the presence of low concentrations of citrate.

Effect of Insulin upon Oxygen Uptake of Muscle Mince—The last column of Table II gives the ratio of the oxygen uptake with insulin to that without insulin at the particular concentration of citrate indicated. This ratio varies from 0.89 to 1.37 and in the mean is 1.13 ± 0.023, which we consider significantly different from 1.0. That is to say, we find that insulin had an effect upon the oxygen uptake of pigeon muscle mince (13 per cent increase) although this is less than that found by Krebs and Eggleston (55 per cent). Whether this effect of insulin is manifested only in the presence of citrate is difficult to say. For the six observations of the effect of insulin without added citrate the mean ratio[2] was as follows:

	No of observations	Ratio, O_2 with insulin to O_2 without insulin
No citrate	6	1.08 ± 0.055
With citrate	21	1.12 ± 0.026

These ratios are not significantly different.

Action of Insulin with Fumarate on Normal and Diabetic Pigeon Muscle Mince—According to the Krebs cycle hypothesis, insulin should be effective in catalyzing oxidations of muscle mince with fumarate as well as with citrate. We tested this (Table III) on normal pigeon breast muscle mince, but were unable to find any consistently significant effects of insulin upon the oxygen uptake. In one case, the addition of hexosediphosphate as a known precursor of triose did not strikingly enhance the action of insulin.

If insulin is a limiting factor in muscle oxidations, it should be

[2] Whenever a mean value together with the standard error of the mean is given, the latter was calculated by the method of Peters; *viz.*,

$$\text{Standard error of mean} = \frac{1.25\ \Sigma\ \text{deviations from mean}}{n\sqrt{n-1}}$$

TABLE II

Effect of Insulin upon Total Oxygen Uptake of Normal Pigeon Muscle Mince in Presence of Citrate at Varying Concentrations at 38°

Boiled sheep heart extract present in all vessels; phosphate Buffer I or II; initial pH 6.8; 0.3 gm. of mince in 3.0 cc.

Pigeon No.	Citrate	Total O_2 uptake, no insulin	Zinc-free insulin	Ratio of O_2 with insulin to O_2 without insulin
	mM per l.	*micromoles per gm.*	*units per cc.*	
36	0	326	1	1.24
	2	476	1	1.07
	30	297	1	1.05
37	0	130	1	1.18
	2	162	1	1.25
	40	104	1	1.37
43	0	201	1	1.05
	2.6	327	1	1.34
	12.4	474	1	1.22
	20.8	520	1	1.28
46	0	215	1	1.04
	0.4	215	1	1.15
	0.8	221	1	1.14
	1.2	258	1	1.14
	1.6	286	1	1.10
	2.0	300	1	1.11
62	4	346	1	1.11
63A	20	407	0.5	1.05
	20	407	1.0	1.12
	30	407	2.0	0.98
63B	20	510	0.5	1.03
	20	510	1.0	1.07
	20	510	2.0	0.89
98	0	200	1	0.90
	10	282	1	0.98
101A	0	159	1	1.07
	4	220	1	1.00
	4*	199*	1	1.02*
101B	0	286	1	0.92
	2	312	1	0.99
	2*	338*	1	1.04*
Mean (31 samples)				1.13 ±0.023

* Also contained 0.005 M hexosediphosphate.

quite effective in accelerating oxygen uptake in the diabetic pigeon. But we did not find this to be the case. Two completely depancreatized pigeons were prepared for us by Dr. I. Ravdin, of the Department of Research Surgery, to whom we are greatly indebted. Both of these pigeons showed within 48 hours a marked elevation of the blood sugar and a considerable elimination of glucose. They were used 48 hours after the operation, when they

TABLE III

Effect of Fumarate with and without Insulin upon Oxygen Uptake of Normal and Diabetic Pigeon Muscle Mince at 38°

0.30 gm. of mince in each vessel; total volume 3.0 cc.; Buffer II; boiled heart extract 0.33 of volume; Lilly's zinc-free insulin at 1 unit per cc.

Pigeon No.	Basic O_2	Fumarate	Ratio of O_2 uptake to basic O_2 uptake	
			No insulin	With insulin
	micromoles per gm.	*mM per l.*		
Normal				
98	200	0		0.90
		5	1.15	1.21
101B	286	0		0.92
		2	1.02	0.96
		2*	0.97*	1.11*
Diabetic				
93A	223	5	0.97	0.98
		5†	0.89†	0.85†
95A	153	0		1.22
		5	1.00	1.18
		5†	1.14†	1.28†
		5‡	0.79‡	1.18‡

* Also contained 0.005 M hexosediphosphate.

† Also contained 0.1 per cent glycogen.

‡ Also contained 0.020 M glucose.

were in excellent shape. The duodenal circulation was quite intact and there was no evidence of necrosis of the gut. No pancreatic remnant was found. The results with these preparations are also shown in Table III. We found with fumarate present in the medium that insulin was no more effective in increasing the oxygen uptake than in the case of the normals.

Action of Insulin upon the Oxygen Uptake of Muscle Mince of

Normal and Diabetic Cat—With normal cat muscle mince we were unable to find any catalytic effect of citrate upon the oxygen uptake. Nor did we find that insulin with citrate was effective. This is in agreement with the findings of Shorr and Barker (1939).

TABLE IV

Effect of Citrate with and without Insulin upon Oxygen Uptake of Muscle Mince of Normal and Depancreatized Cats at 38°

0.30 gm. of muscle; total volume 3.0 cc.; Buffer II; boiled heart extract 0.33 of volume; Lilly's zinc-free insulin, 1 unit per cc.

Cat No.	Basic O_2		Ratio of O_2 uptake to basic O_2 uptake: No insulin	Ratio of O_2 uptake to basic O_2 uptake: With insulin
		Normal cat		
	micromoles per gm.			
60	60	No addition		
		0.004 M citrate	1.13	
		0.1 % glucose	0.87 ± 0.04*	0.80 ± 0.005*
		0.1 % " + 0.004 M citrate	0.97 ± 0.003*	1.03 ± 0.06*
		Depancreatized cats		
52	50	No addition		
		0.002 M citrate	1.25 ± 0.02*	1.57 ± 0.16*
		0.010 " hexosediphosphate	1.52	1.53
		0.002 " citrate + 0.01 M hexosediphosphate	1.76 ± 0.09*	1.86 ± 0.02*
55	19	0.009 M hexosediphosphate		
		0.009 " " + 0.002 M citrate	1.02	1.01
55	9.5	0.1 % glucose		
		0.1 % " + 0.002 M citrate	1.35 ± 0.05*	1.33 ± 0.03*

* Mean of two samples.

In the case of the diabetic cat we found considerable variation in the basic oxygen uptake of the muscle mince. However, we found that citrate or citrate + hexosediphosphate increased the oxygen uptake, but this increase is considerably less than the oxygen equivalent of the citrate added. With insulin + citrate, the respirations were essentially the same as those without insulin;

i.e., we were unable to find any catalytic effect of insulin upon oxygen uptake with diabetic cat muscle mince. It will be noted (Cat 55) that insulin in the presence of glucose and citrate did not enhance the respiration. See Table IV.

Effect of pH and Proportion of Boiled Muscle Extract—Krebs and Eggleston state that the catalytic effect of citrate is found

TABLE V

Respiration of 0.30 Gm. of Muscle Mince from Normal Fasted Pigeon (No. 1010) Suspended in 100 Per Cent of a 1 : 1 Boiled Beef Heart Extract, and Effect of Citrate with and without Insulin Thereon

Final composition of buffer (with or without extract), Na_2HPO_4 0.050 M, NaCl 0.133 M, KCl 0.007 M, $MgCl_2$ 0.005 M. Initial pH 7.00 (buffer without mince); final volume 3.0 cc.; 38°.

Lilly's zinc-free insulin = 1 unit per cc.; Stearn's zinc insulin = 1 unit per cc. Basic oxygen uptake with extract = 260 micromoles per gm. for 150 minutes.

	Ratio of O_2 uptake to basic O_2 uptake	Ratio of O_2 with insulin to O_2 without insulin in class	Final pH
No extract; no additions	0.50		6.78
With 100% extract			
No additions	1.00		6.81
Insulin	1.00	1.00	6.74
0.001 M citrate	1.06 ± 0.03*		6.81 ± 0.01*
0.001 " " + insulin	1.13 ± 0.07*	1.07 ± 0.05*	6.81 ± 0.01*
0.001 M citrate + zinc insulin	1.17	1.12	6.83
0.005 M citrate	1.34		6.88
0.005 " " + insulin	1.17	0.87	6.88
0.005 M citrate + zinc insulin	1.00	1.00	6.83

* Mean of duplicate samples.

only at pH 6.8 and not 7.2 and attributed Breusch's (1937) failure to find it to his use of buffers at 7.2. We have paid particular attention to this point. Besides using buffers initially at pH 6.8, we have determined the pH of the final equilibrated mince with the glass electrode. We have never found a final value lower than 6.6 pH and in the mean the values center around 6.7.

We have also tested more specifically the relation of the insulin

effect to the concentration of coenzymes and substrates contained in the boiled heart extract. For this purpose we used 100 per cent of a 1:1 boiled extract of beef heart which has been made up to the concentrations indicated in Table V by the addition of the solid salts. The presence of the boiled extract doubled the total oxygen uptake, indicating that it was rich in coenzymes and substrates. In no respect are the results of this experiment different from those previously discussed. We found an effect of citrate at 0.001 M and 0.005 M equal only to 34 per cent and 39 per cent respectively of the oxygen equivalent of citrate added. The effects of insulin varied from −13 to +12 per cent. At the end of the respiratory period the pH of the contents of the Warburg vessels was determined by means of the glass electrode. The values centered around 6.8 pH, at which, according to Krebs and Eggleston, the citrate effects should be maximum.

DISCUSSION

The evidence that insulin is an obligatory catalyst for the oxidation of carbohydrate by the muscles is not unequivocal. The classical view based on experiments in the intact animals, upon eviscerated preparations, or perfused hearts maintains the affirmative, but recent developments, particularly those related to the Houssay phenomenon, have considerably shaken this opinion. The demonstration of a catalytic effect of insulin on the carbohydrate metabolism of muscle by experiments *in vitro* is, for obvious reasons, of prime importance. Krebs and Eggleston have shown that insulin enhances the oxygen uptake of minced pigeon muscle. In our own experiments we have shown the same thing. The effect is small, but, in our opinion, it is undoubtedly significant. We therefore come to the conclusion of Krebs and Eggleston; *viz.*, insulin catalytically increases the oxygen uptake of minced pigeon muscle. The relation of this effect of insulin to the carbohydrate metabolism of muscle is still obscure.

Krebs' citrate cycle hypothesis, and the relation of the effect of insulin to it, remains a matter for future experimentation. Breusch (1937) was unable to confirm Krebs and Johnson (1937) in a crucial aspect of the problem; *viz.*, the enzymatic synthesis of citrate from oxalacetate by muscle mince. Nor was he able to find a catalytic effect of citrate as determined by the excess of

oxygen uptake over the oxygen equivalent of the citrate added. In our own experiments, we have been unable to show, by the same criterion, any catalytic effect of citrate upon the respiration of muscle mince of normal or diabetic pigeons and of normal or diabetic cats.

The relation of citrate to the catalytic action of insulin remains undecided. In our experiments with normal animals we could find no difference between the effect of insulin with or without citrate. We were also unable to demonstrate an enhanced effect of insulin, either with citrate or fumarate, upon the respiration of the muscle mince of the diabetic pigeon or cat. Our results are, in general, in conformity with those of Shorr and Barker (1939) who found small (20 per cent) but significant effects of insulin upon the respiration of pigeon muscle mince. They, also, were unable to demonstrate this effect with muscles of normal cats, or with dogs, or rabbits.

If insulin is a limiting factor in the oxidations in muscle, one might expect an increased effect of insulin in the diabetic. The failure to observe such an enhancement by insulin upon the oxygen uptake of muscle mince of diabetic pigeons and cats is a serious defect in the evidence for the hypothesis. But this must not be weighed too heavily, for our experience brings us to the conclusion that the penetration of insulin into tissues, or the development of its action, requires a considerable time. The respiration of muscle mince falls off rapidly to 0 in 2 to 3 hours, which time may be quite insufficient for insulin to exert its full effect. This point is illustrated in a subsequent paper (Stadie, Zapp, and Lukens, 1940) in which considerable effects of insulin upon the ketone formation by diabetic cat liver slices were manifested after equilibration for 4 hours.

We wish to express our thanks to Mildred S. Wright for her helpful criticisms and assistance in the experiments.

SUMMARY

1. The oxygen consumption of muscle mince of normal pigeon breast suspended in phosphate buffer at pH 6.8 together with boiled muscle extract was increased (mean 43 per cent) by the presence of citrate.

2. This effect, in our opinion, is not catalytic, since the excess oxygen is always less than the oxygen equivalent of the citrate added.

3. Insulin at 1 unit per cc. increased the oxygen uptake of normal pigeon muscle mince (mean 13 per cent), but this effect is apparently independent of the presence of citrate.

4. The effect of insulin was not significantly enhanced when muscle mince of diabetic pigeons or cats was used.

5. The relation of these findings to Krebs' citrate cycle hypothesis is discussed.

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THE EFFECT OF INSULIN UPON THE KETONE METABOLISM OF NORMAL AND DIABETIC CATS*

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Until recently the views prevalent in the literature with respect to ketone formation in the normal and diabetic organism were as follows: The long fatty acid carbon chains are split by successive β oxidation, 2 carbons at a time, with the formation of intermediate shorter fatty acids and acetic acid (Knoop hypothesis). Normally this process continues to completion, but in the absence of sufficient carbohydrate stores (fasting), or without insulin as an obligatory carbohydrate catalyst (diabetes), the process stops at the 4-carbon ketone stage. Each molecule of fatty acid gives rise to 1 molecule of ketone. An obligatory chemical coupling of the oxidation of carbohydrate and ketones was assumed. Failing carbohydrate oxidation, ketones cannot be utilized by the peripheral tissues; hence they are excreted *in toto*.

Ketone Utilization by Peripheral Tissues—Since 1928, however, considerable evidence has accumulated in the literature, necessitating complete revision of these concepts. For example, Snapper and Gruenbaum (1928), in perfusion experiments on striated muscle of normal animals, found a considerable disappearance of circulating ketones. Chaikoff and Soskin (1928), from a study of the rate of disappearance of ketones from the blood following injection of acetoacetate, concluded that ketones are utilized by the muscles of the diabetic as well as the normal eviscerated dog. Later Friedemann (1936) and Mirsky and Broh-

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Kahn (1937) performed similar experiments and came to the same conclusion. Barnes, MacKay, Moe, and Visscher (1938) demonstrated a disappearance of β-hydroxybutyrate from heart-lung preparations of normal dogs and goats. Recently Blixenkrone-Møller (1938, *a*, *b*) showed in a convincing manner, by perfusion experiments on the hind limbs of normal and diabetic cats, that there was an active utilization of ketones by muscle. This was markedly increased in the contracting muscle, and Blixenkrone-Møller (1938, *b*) concluded that ketone oxidation might furnish a considerable fraction of the total energy requirements. Toeniessen and Brinkman (1938), from similar perfusion experiments in the normal rabbit, also concluded that ketone bodies formed in the liver are burned in the muscles.

Hypothesis of Multiple Alternate Oxidation of Fatty Acids—As early as 1916, Hurtley rejected the Knoop hypothesis of successive β oxidation as an explanation of the mechanism of production of ketones in the diabetic. Large amounts of the intermediate fatty acids (butyric, caproic, etc.) should be formed in a diabetic excreting 70 gm. of ketones a day. But Hurtley found no significant amounts of butyric acid in the blood or tissues in such a case. He therefore proposed the hypothesis that the fatty acid is attacked at alternate carbon atoms simultaneously along the whole length of the carbon chain according to the scheme, $\ldots CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \ldots = \ldots CO \cdot CH_2 \cdot CO \cdot CH_2 \cdot CO \cdot CH_2 \ldots$

Jowett and Quastel (1935) studied the rate of formation of ketones from fatty acids by liver slices. They also abandoned the successive β oxidation hypothesis and adopted that of Hurtley, which they called the "multiple alternate oxidation" hypothesis. According to it, the fatty acids undergo an oxidation throughout the entire fatty chain, alternate carbon atoms being affected. As a result, the entire molecule breaks down into acetoacetic acid and perhaps other acid products.

Deuel, Hallman, Butts, and Murray (1936) studied the rate of excretion of ketone bodies in rats after feeding ethyl esters of fatty acids. Their results indicated that in the case of palmitic, stearic, and oleic acids at least 3 molecules of ketones were formed per molecule of fatty acid oxidized, and they therefore supported the hypothesis of multiple alternate oxidation.

Blixenkrone-Møller (1938, *a*) compared the oxygen consumption

of perfused livers from diabetic cats with the total ketone production. It was possible to explain the low oxygen to ketone ratio only by assuming that 4 molecules of ketones were formed per molecule of fatty acid oxidized.

In the experiments reported in this paper we present evidence bearing on these problems. We have determined the rate of formation of ketone bodies and the oxygen consumption of surviving liver slices of normal and diabetic cats. We have also determined directly the utilization of ketones by muscle mince of normal and diabetic cats. In addition, we have experiments showing the influence, *in vitro*, of carbohydrates with and without insulin upon ketone formation by the liver of diabetic cats.

EXPERIMENTAL

Animals—The diabetic cats were depancreatized under nembutal anesthesia and used, as a rule, 48 hours later. The completeness of pancreatectomy was confirmed by autopsy and by the degree of glycosuria.

Preparation of Tissue. Liver—After a preliminary period of 1.0 hour (for the collection of urine) the abdomen was opened under nembutal anesthesia, and the aorta was severed. The liver was placed in ice-cold saline and weighed. Samples were taken for total fats, glycogen, ketones, etc. Slices (0.40 ± 0.05 mm.) were prepared with a double razor blade cutter. Averaging 200 mg., they were placed in conical Warburg vessels together with 3.0 cc. of appropriate medium and 100 per cent oxygen. The respiration period was 2.0 hours. The total oxygen consumption and CO_2 production determined by the customary Warburg technique (38°) are given as micromoles per gm. of wet liver.

Muscle—Portions of muscle as free as possible from tendinous tissue were quickly dissected out, collected on cracked ice, and minced (in the cold room at 0°) by the Latapie mincer. Weighed portions of this mince were transferred to Warburg vessels containing the appropriate media, through which 100 per cent O_2 was passed for 1 minute, and transferred to the bath at 38° for measurement of oxygen consumption, etc.

Collection of Urine—The urinary ketone excretion was always measured exactly 1.0 hour before the animals were killed. The bladder was initially emptied by pressure upon the abdomen.

At the end of 1.0 hour, the bladder was again emptied in the same way, and the completeness of emptying was verified by inspection of the bladder after the abdomen was opened.

Buffered Medium—This was always 3.0 cc., and the composition (aside from additions indicated for the individual experiments in the tables) was 0.050 M sodium phosphate (pH 7.0), 0.130 M NaCl, 0.007 M KCl, and 0.005 M $MgCl_2$.

Insulin[1]—The insulin used was Lilly's amorphous insulin of highest purity. It was zinc-free and assayed 22 units per mg. Appropriate solutions were made up at pH 7.0 and added to the vessels at the final concentration indicated.

Analysis of Ketones. Tissue and Warburg Vessel Contents—At the end of the period of equilibration, the Warburg vessels were chilled in ice water for 5 minutes and then, through the side sac, 0.5 cc. of a 2 per cent solution of sodium bisulfite was added to bind any acetone which had formed. The tissue plus the contents of the vessel was transferred quantitatively to a calibrated centrifuge tube and appropriate amounts of tungstic acid reagent were added to precipitate the proteins. Replicates of the centrifugate were then analyzed for ketones by a slightly modified Shipley and Long (1938) method, which we found excellently suited for the determination of amounts of ketones down to 0.05 to 0.1 micromole. Each sample was analyzed separately for acetoacetic acid and β-hydroxybutyric acid.

The determination of the acetone in the distillates was carried out by means of the Scott-Wilson reagent. Readings of the turbidity were made on the Evelyn photoelectric colorimeter, with a standard curve which was measured anew for each set of determinations.

All values of acetoacetate, β-hydroxybutyrate, or total ketones are reported as micromoles per gm. of wet tissue.

Calculation—To calculate from gm. of liver to kilos of cat we used our average value of 26.3 ± 1.5 gm. of liver per kilo of cat. To calculate from gm. of muscle to kilo of cat we assumed an average of 350 gm. of muscle per kilo of cat.

Ketone Formation by Liver Slices of Fasted Normal Cats—There was found (Table I) a considerable ketone formation by the liver. Since urinary excretion was zero in all cases during the pre-

[1] We are indebted to Dr. F. B. Peck of the Lilly Research Laboratories for generous supplies of insulin.

experimental period of 1.0 hour, we conclude that there was a considerable ketone utilization (240 ± 17 micromoles per kilo of cat per hour)[2] by the peripheral tissues, presumably for the most part by the muscles.

TABLE I

*Basic Ketone Formation by Liver Slices and Ketone Utilization by Normal and Diabetic Cats**

Average weight of slices 200 mg.; 3.0 cc. of phosphate buffer (no added nutrient); 2.0 hours equilibration; 38°.

Cat No.	Fasted	Cat weight	Liver weight	Ketone formation: Per gm. liver per hr.	Ketone formation: Per kilo cat per hr.	Ketone excretion per kilo cat per hr.	Ketone utilization per kilo cat per hr.
				Normal cats			
	hrs.	*kg.*	*gm.*	*micromoles*	*micromoles*	*micromoles*	*micromoles*
99A	48	3.5	104	8.2 ± 2.9	240 ± 86	0	240 ± 86
99B	72	2.0	43	13.3 ± 1.0	280 ± 21	0	280 ± 21
99C	84	2.6	68	5.7	150	0	150
Mean				9.7 ± 1.4	240 ± 17	0	240 ± 17
				Diabetic cats (48 hrs. after pancreatectomy)			
31	48	2.6	64	36	885	330	655
79	48	3.1	66	50	1065	105	960
96A	48	2.8	84	70	2105	155	1950
96B	48	2.1	62	55	1175	30	1145
96C	48	2.5	74	27	810	0	810
96D	48	3.1	77	33	825	30	795
96E	48	2.4	58	53	1280	185	1095
96F	48	2.7	82	55	1650	180	1470
Mean				47 ± 6	1200 ± 150	130 ± 35	1100 ± 150

* Whenever in this or the subsequent tables a mean value with its standard error is given, it indicates that two or more separate samples of tissue were equilibrated and the mean value recorded.

Basic Ketone Formation by Liver Slices from (48 Hour) Diabetic Cats—The average ketone formation by the liver (1200 ± 150

[2] Wherever a mean value together with the standard error of the mean is given, the latter was calculated by the method of Peters; *viz.*,

$$\text{Standard error of mean} = \frac{1.25\ \Sigma\ \text{deviations from mean}}{n\sqrt{n-1}}$$

micromoles per kilo of cat per hour) during the experimental period *in vitro* was about 10 times the mean urinary excretion during the 1.0 hour preexperimental period *in vivo.*

In the diabetic cat, in contrast with the normal cat, the enzyme system concerned with the oxidation of ketones may be assumed to be saturated when ketones are excreted, and ketone utilization is then probably maximal. The mean excess (1100 ± 150 micromoles per kilo per hour) of ketone formation over ketone excretion probably represents the maximum ketone utilization of the diabetic cat.

These experiments illustrate further that the urinary ketone excretion of normal or diabetic animals is unreliable as an index of the rate of ketone production.

Direct Determination of Ketone Utilization by Minced Muscle from Normal and Diabetic Cats- We were successful in demonstrating this in the case of acetoacetate in the following way. 0.2 gm. samples of muscle mince of normal or diabetic cats were equilibrated for 2.0 hours at 38° in Warburg vessels containing 3.0 cc. of phosphate buffer and known amounts of acetoacetate. Parallel samples of muscle mince were run without the addition of acetoacetate to measure the spontaneous formation of β-hydroxybutyrate which usually occurred, and for which a correction was made in the calculation. At the end of the period of equilibration, the samples were deproteinized and analyzed for acetoacetate and β-hydroxybutyrate. The muscle mass was assumed to be 350 gm. per kilo of cat and ketone utilization per kilo of cat was calculated accordingly from the value per gm. of muscle. By this direct method we found (Table II) a utilization of acetoacetate, which in the mean was 915 ± 265 micromoles per kilo per hour.

In similar experiments with added β-hydroxybutyrate, we were unable to demonstrate a diminution of total ketones (Table II). In point of fact, we found as a rule an increase, chiefly of β-hydroxybutyrate. There is evidence in the literature against the participation of the muscles in ketone formation. On the other hand, Krebs and Johnson (1937) have demonstrated the formation of ketones in muscle from pyruvic acid, implying that carbohydrate as well as fat may be a source of ketones. Our own experience and that of Krebs and Johnson indicate that it is not possible to exclude the muscles as a site of ketone formation.

Our experiments also raise the question whether acetoacetate is the sole form of ketone which is oxidized by muscle.

Ketone Utilization by Diabetic Minced Muscle Simultaneously Equilibrated with Diabetic Liver Slices—It is possible that the oxidation by muscle mince of β-hydroxybutyrate is limited to the naturally occurring *l* isomer. This we did not have available.

TABLE II

Direct Determination of Utilization of Ketones by Muscle Mince of Normal and Diabetic Cats

200 mg. of mince; 3.0 cc. of phosphate buffer with added ketones; 2.0 hours; 38°.

Cat No.	Class	Concentration of acetoacetic acid	Net change* of ketones per kilo cat per hr.
		mM per l.	*micromoles*
96A	Diabetic	5.0	−1600 ± 60
96C	"	0.4	−570 ± 210
99A	Normal	2.0	−1580 ± 175
99B	"	2.0	−315 ± 0
99C	"	0.4	−100
Mean (9 samples)			−915 ± 265
		β-Hydroxybutyric	
96A	Diabetic	5.0	−730 ± 420
96B	"	2.0	+670 ± 210
96C	"	0.4	−130
99A	Normal	2.0	+600 ± 700
99B	"	2.0	+350 ± 140
99C	"	0.4	+90
Mean			+140 ± 220

* Calculated from the change per gm. of muscle by assuming the muscle mass to be 350 gm. per kilo.

At any rate, we were able to demonstrate the oxidation of ketones by diabetic muscle mince by a third method; *viz.*, the simultaneous equilibration of diabetic liver and muscle. By this device the muscle would presumably be supplied with the natural ketones continuously formed by the diabetic liver and, to a certain extent, the conditions *in vivo* would be imitated. 0.2 gm. portions of diabetic muscle mince were placed in Warburg vessels together

with 30 mg. slices of diabetic cat liver and 3.0 cc. of phosphate buffer. 100 per cent O_2 was passed into the vessels for 1 minute and the vessels equilibrated at 38° for 2.0 hours. In parallel vessels, similar portions of muscle and liver were equilibrated independently. The assumption was made that the rate of formation of ketones by the liver equilibrated with the muscle was the same as that of the liver equilibrated independently, and the calculations were made accordingly. We found (Table III) by this method a mean utilization of ketones of 2070 ± 430 micromoles per kilo per hour.

TABLE III

Ketone Utilization by Muscle Mince of Diabetic Cat When Simultaneously Equilibrated with Diabetic Liver Slices As Source of Ketones

200 mg. of muscle mince; 30 mg. of liver slices; 3.0 cc. of phosphate buffer; 2.0 hours; 38°.

Cat No.	Utilization of ketones per kilo cat per hr.	O_2 consumption of liver + muscle per 2.0 hrs.	
		Calculated	Observed
	micromoles	*micromoles*	*micromoles*
96A	2800	64	65
96B	1820	31	34
96C	1580	34	33
Mean... ...	2070 ± 430		

We also compared the observed oxygen uptake of the combined liver and muscle systems with that calculated from the oxygen uptake of the samples equilibrated independently. The agreement of the calculated with the observed values is an indication that the oxidations of the liver slice and muscle mince in the composite system were going on independently of each other.

Comparison of Portal and Hepatic Blood Ketones—To show that the rate of ketone formation by liver slices *in vitro* is essentially the same as that of the intact liver with normal circulation, we obtained blood samples simultaneously from the portal and hepatic veins of diabetic cats under nembutal anesthesia. These samples were analyzed for ketones.

The blood flow through the diabetic liver is quite variable, but as a sufficient approximation we can take the mean value de-

termined by Schmid (1908) for the cat as 32 cc. per hour per gm. of liver. By the equation

$$\frac{\text{Gm. liver}}{\text{Kilos of cat}} \times \text{liver blood flow (cc. per gm. per hr.)}$$

$$\times \text{ portal-hepatic difference (micromoles per cc.)}$$

$$= \text{ ketone formation (micromoles per kilo of cat per hr.)}$$

we calculated the rate of ketone formation *in vivo* (Table IV). The mean value of 1265 ± 410 micromoles per kilo per hour is to be compared with our mean value obtained *in vitro* of 1200 ± 150 micromoles per hour (Table I). The identity of these values

TABLE IV

Ketone Formation by Liver of Diabetic Cats, Calculated from Portal and Hepatic Blood Ketone Concentration in Intact Animal

Cat No.	Blood ketones		Calculated liver ketone formation per kilo cat per hr.
	Portal	Hepatic	
	micromoles per cc.	*micromoles per cc.*	*micromoles*
96D	9.4	10.5	875
96E	16.4	17.7	1000
102A	9.7	11.7	1940
Mean .			1265 ± 410

is evidence that the rate of ketone formation by liver slices *in vitro* is the same as the rate *in vivo*.

We have calculated (Table V) from the data in the literature the values for ketone utilization obtained by different workers and different methods. These are in such good agreement that we can accept with assurance the value of 1300 micromoles per kilo per hour as representing the maximum basal ketone utilization.

Molecular Ratio of Ketones Formed to Fatty Acid Oxidized by Liver of Diabetic Cats; Multiple Alternate Oxidation Hypothesis—It is possible to make an estimate of the ratio from a knowledge of the molecular ratio of the oxygen uptake to the ketone formation. Three cases have to be considered.

1. If one assumes, according to the older views, that fatty acids

TABLE V

Determinations in Literature of Ketone Utilization by Peripheral Tissues of Normal and Diabetic Animal

	Mean ketone utilization
	micromoles per kg. per hr.
Chaikoff and Soskin (1928)	
By measurement of rate of disappearance of injected ketones from blood of	
Diabetic eviscerated dogs	1200 ± 400
Normal eviscerated dogs	1400 ± 230
Blixenkrone-Møller (1938, *b*)	
By comparison of ketone formation by perfused diabetic cat livers with prior urinary ketone excretion	1600 ± 330
By measurement of rate of disappearance of ketones from blood perfused through	
Resting hind limbs of normal and diabetic cats	1100 ± 160
Working " " " " " " "	5400 ± 280*
Stadie, Zapp, and Lukens	
By comparison of ketone formation by diabetic cat liver slices with prior urinary ketone excretion	1100 ± 150
By measurement of ketone utilization of normal and diabetic cat muscle mince in presence of added acetoacetate	915 ± 265
By measurement of ketone utilization by diabetic cat muscle mince simultaneously equilibrated with diabetic cat liver slice	2070 ± 430
By measurement of the portal-hepatic ketone difference of diabetic cats	1240 ± 410
Mean basal ketone utilization	1340 ± 130

* This value is excluded from the basal mean.

in the diabetic are oxidized by β oxidation to the ketone stage only, the reaction for the oxidation of a typical fatty acid (palmitic) is

$$C_{16}H_{32}O_2 + 18.5O_2 = C_4H_8O_3 + 12CO_2 + 12H_2O$$
$$\text{Molecular ratio of } O_2 \text{ to ketones} = 18.5:1; \text{ R.Q.} = 0.65$$

2. It is possible, however, that the oxidation in the liver is partial, yielding only 1 molecule of ketone, the balance of the molecule being oxidized to some other acid products, perhaps acetic acid. In that case, the reaction is

$$C_{16}H_{32}O_2 + 6O_2 = C_4H_8O_3 + 6CH_3COOH$$
$$\text{Molecular ratio of } O_2 \text{ to ketones} = 6:1; \text{ R.Q.} = 0$$

3. According to the multiple alternate oxidation hypothesis, the entire molecule is simultaneously oxidized, yielding 4 molecules of ketones; the reaction is then

$$C_{16}H_{32}O_2 + 5O_2 = 4C_4H_6O_3$$

Molecular ratio of O_2 to ketones = 1.25:1; R.Q. = 0

In Table VI we give the observed values for the oxygen uptake and the ketone formation by liver slices of diabetic cats. In the mean, the molecular ratio of these two values is 1.68 ± 0.12. It must be remembered that there are oxidations other than ketone

TABLE VI

Molecular Ratio of Oxygen Uptake to Ketone Formation by Liver Slices from Diabetic Cats

200 mg. slices; 3.0 cc. of phosphate buffer; 2.0 hours; 38°.

Cat No.	O_2 per gm. per 2.0 hrs.	Ketone formation per gm. per 2.0 hrs.	Ratio, O_2 to ketones	R.Q.
	micromoles	*micromoles*		
79	157 ± 3	100	1.57	0.21
96A	192 ± 18	138	1.39	0 33 ± 0.04
96B	201	110	1.83	0.39
96C	163	110	1.48	0.40
96D	149	67	2.22	0.40
96E	177	106	1.70	0.22
Mean	175 ± 8	105 ± 8	1.68 ± 0 12	0.32 ± 0.04

formation occurring in the liver which, if corrected for, would make the ratio still lower. For example, we have previously determined (Stadie, Lukens, and Zapp, 1940) that the mean rate of deamination by diabetic liver slices is 16.4 micromoles of urea per gm. per 2.0 hours. It is quite permissible, then, to correct the total oxygen uptake by this amount, in which case the mean ratio of O_2 to ketones would be 1.54:1. This ratio is sufficiently close to the one calculated according to the hypothesis of multiple alternate oxidation to warrant the conclusion that, in the diabetic cat liver, each fatty acid molecule on oxidation yields 4 molecules of ketones. This is in complete agreement with the conclusion of Blixenkrone-Møller (1938, *a*) on the basis of his experiments

on the rate of oxygen consumption and ketone formation in perfused diabetic cat livers.

It is to be noted further that the low R.Q. (0.32 ± 0.04) of diabetic cat liver slices is in accordance with the expectation that the major portion of the oxidations occurring in the diabetic liver do not produce CO_2.

Effect of Fructose with and without Insulin upon Ketone Formation by Diabetic Cat Liver—Our experiments (Table VII) show the possibility of markedly influencing the ketone formation by diabetic liver slices with fructose with and without insulin. Most

TABLE VII

Effect of Insulin with and without Substrates upon Ketone Formation by Liver Slices from Diabetic Cats

200 mg. slices; phosphate buffer, 3.0 cc.; Lilly's Zn-free insulin, 1 unit per cc.; 2.0 hours; 38°.

Cat No.	Basic ketone formation, no added nutrient, per gm. per 2.0 hrs	Per cent change of basic ketone formation						
		Insulin only	10 mM fructose		10 mM fructose + 0.002 M fumarate		0.002 M fumarate	
			No insulin	With insulin	No insulin	With insulin	No insulin	With insulin
	micromoles							
79	100	+11						
96B	117		−12	-39				
96C	55		−20	−27	−49	−62		
96D	67	+11	+6	+17	+5	−15	−36	−26
96E	106	+5	−22	−49	−15	−50	−17	−27
Mean		+9	−12	−25	−20	−42	−26	−26

marked is the effect with fructose + fumarate + insulin (−42 per cent), a finding in accordance with the hypothesis of Szent-Györgyi (1937) that the 4-carbon dicarboxylic acids may inhibit the formation of ketones in the diabetic liver.

Effect of Time of Equilibration upon Action of Insulin on Ketone Formation of Diabetic Cat Liver Slices The inhibiting action of insulin upon the ketone formation of diabetic cat liver slices can be considerably enhanced if a longer time than 2 hours is allowed for its action. We equilibrated three different samples of liver slices from the same diabetic cat. The phosphate buffer contained

(*a*) no additions, (*b*) 0.010 M fructose, or (*c*) 0.010 M fructose + 1 unit per cc. of insulin. At intervals of 1 hour the slices were transferred to similar fresh media. The media from each respiratory period were then analyzed for total ketones. The summations of the ketone formation are plotted in Fig. 1. In all cases the oxygen uptake continued essentially at the initial rate for 4 hours. This indicated the continued viability of the liver cells. The progressive fall in the rate of ketone formation is greatest in

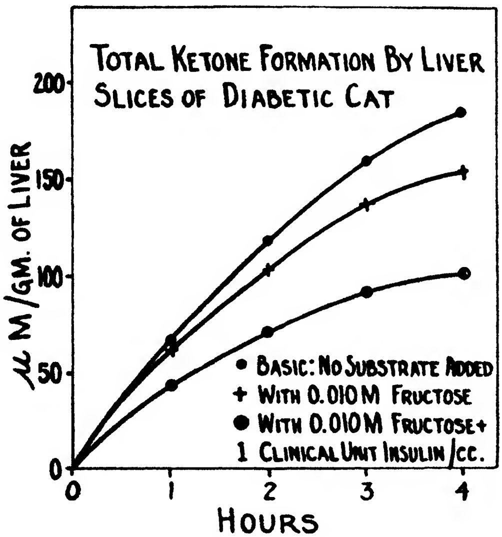

FIG. 1

the case of fructose + insulin, and at the end of the 4 hour period the ratios of ketone formation in the three cases were 1:0.87:0.55.

Ketone Formation by Liver Slices from Diabetic Cat Previously Injected With Insulin.—The reversal toward normal of the diabetic type of metabolism of the isolated liver slice may also be accomplished by the prior injection of adequate doses of insulin, provided sufficient time elapses after the injection. For this purpose we used cats 48 hours after pancreatectomy. Initial samples of blood from the ear vein were taken for glucose and ketones. The cats were then injected subcutaneously with divided doses of Lilly's amorphous insulin, and frequent blood

sugar determinations were made by the Shaffer-Hartmann-Somogyi method (Peters and Van Slyke, 1932) until the desired level was reached. We found by experience that doses of 30 to 40 units of insulin and a lapse of 4 to 6 hours were necessary in order to accomplish this. The cats were then anesthetized with nembutal as in our previous experiments, and liver slices were prepared and equilibrated for 2.0 hours in phosphate buffer which contained no additional insulin. In some cases substrates were added. At the end of the period, the contents of the Warburg

TABLE VIII

Ketone Formation by Liver Slices from Diabetic Cats Following Injection of Insulin

Cat No.	Cat weight	Liver weight	Blood sugar		Insulin given	Insulin action	Basic ketone formation*		Per cent change with		
			Initial	Final			Per gm. liver per hr.	Per kilo cat per hr.	10 mM fructose	10 mM glucose	10 mM *d*-lactate
	kg.	*gm.*	*mg. per cent*	*mg. per cent*	*units*	*hrs.*	*micromoles*	*micromoles*			
102A†	2.7	82	264	194	6	1.5	53 ± 14	1650 ± 440	−19	−15	−32
102B	2.6	79	243	138	40	5.8	17 ± 2	495 ± 40	0	+13	+6
102C	2.1	56	226	60	35	6.0	17 ± 0.5	460 ± 7	−32	0	−39
102D	1.9	46	338	110	30	4.5	18 ± 4.5	435 ± 110	−22	−35	−49
Mean . .							17 ± 1	460 ± 18	−18	−9	−28

* Values in this column are means of two separate samples of liver slices.

† Excluded from the mean of this series because the time of action and amount of insulin were insufficient.

vessels and the tissue were analyzed for total ketones. The data are presented in Table VIII.

We emphasize the following points. (1) When the doses of insulin or the time elapsed was small, the subsequent rate of formation of ketones (Cat 102A) was not less than the mean value (46 ± 6 micromoles per gm. of liver per hour) found in the cases of untreated diabetic cats. However, the effect of added substrates, *e.g.* fructose, glucose, or *d*-lactate, in diminishing ketone formation is quite apparent, being as much as −32 per cent in the last instance. (2) With adequate dosage of insulin and sufficient time, the effect of the insulin is quite marked. The mean rate of

ketone formation of the three treated cases is 17 ± 1 micromole per gm. of liver per hour or 37 ± 4 per cent of that of untreated cases. (3) The additional effect of the added substrates is also evident; ketone formation was further reduced (with two exceptions) by 20 to 50 per cent. (4) The restoration toward a normal rate of ketone formation may precede the storage of glycogen in the liver and the decrease of the usual high liver fat. In Cat 102B the liver glycogen was 0.05 per cent and the liver fats 18 per cent, essentially that of untreated cats. In Cat 102C, in which the blood sugar was allowed to fall to quite low levels (60 mg. per cent), the liver glycogen was raised to 3.2 per cent. Nevertheless the ketone formation in these two cases was found to be essentially the same. We take this to mean that insulin

TABLE IX

Ketone Formation by Liver Slices from Houssay Cat

Cat No.	Hypophysectomy	Pancreatectomy	Experiment	Cat weight	Liver weight	Blood sugar	Liver glycogen	Liver ketone formation per kilo cat per hr.
	1939	*1939*	*1939*	*kg.*	*gm.*	*mg. per cent*	*per cent*	*micromoles*
105A	June 8	June 14	June 19	3.4	50	60	0.04	68
105B	Sept. 7	Sept. 18	Sept. 20	2 1	49		0.02	104

may act independently to suppress ketone formation and to aid in the storage of glycogen. The view that the storage of glycogen is an obligatory first step in the suppression of ketone formation is not in accordance with these observations.

Ketone Formation by Liver of Houssay Cat—The amelioration of the diabetic status in the hypophysectomized-depancreatized cat is too well known to require comment. This reversal was strikingly demonstrated by a study of the ketone formation by the liver slices of two Houssay cats (Table IX). From the data it is seen that the ketone formation of the liver is less than normal. This experiment emphasizes the possibility that insulin does not react directly in a regulatory fashion with the enzyme system concerned in the oxidation of fatty acids, but indirectly by acting as antagonist to the ketogenic hormone of the pituitary.

Total Respiration and Non-Ketone Respiration of Liver of Normal and Diabetic Cats—We have previously discussed the evidence indicating that the oxidation of a typical fatty acid (palmitic) in the liver follows the equation

$$C_{16}H_{32}O_2 \text{ (palmitic acid)} + 5O_2 = 4C_4H_8O_3 \text{ (}\beta\text{-hydroxybutyric acid)}$$

We may therefore assume that, on the average, 1.25 moles of oxygen are required for the formation of 1 mole of ketone. If fatty acids are the sole precursors of ketones in the liver, it is possible to correct the *total* oxygen consumption of liver slices by subtracting the oxygen required for ketone formation, (*i.e.*, 1.25 × ketones formed) to obtain the *non-ketone* oxygen consumption. Since ketone formation is presumably not accompanied by CO_2 formation, the total CO_2 divided by the non-ketone O_2 gives the *non-ketone* R.Q. These calculations for our series of normal cats, untreated diabetic cats, and insulin-treated cats show some contrasting aspects (Table X).

The mean oxygen consumption of the untreated diabetic cat liver slices was significantly higher than the normal mean. In the treated cats the mean oxygen consumption was restored toward normal.

The chief function of the diabetic liver is the partial oxidation of fatty acids to ketones: only 42/185 or 22 ± 5 per cent of the total oxygen uptake is concerned with oxidations other than ketone formation. This is in sharp contrast with livers from normal fasted and treated diabetic cats, in which 86 ± 8 per cent and 77 ± 3 per cent respectively of the total oxygen consumption are thus concerned.

In the diabetic liver the non-ketone respiration is associated with a high respiratory quotient (1.36 ± 0.34). The significance of this is a matter of conjecture.

In the treated diabetic cases there are indications that the metabolic status of the liver slice is intermediate between the normal and the untreated. For example, the ketone formation is low, the non-ketone oxygen is essentially normal, but the CO_2 is not correspondingly elevated; *i.e.*, the non-ketone R.Q. is low. We have no further experimental data to aid in interpreting the significance of these findings. Our surmise, however, is that in the livers from insulin-treated diabetic cats partial oxidations of fatty

acids still make up the major portion of the hepatic metabolism, but that the oxidations do not produce ketones.

Possibility of Production of Carbohydrates from Fats by Diabetic Liver—The overproduction hypothesis of diabetes postulates that fats in large quantities are converted by the liver into carbohydrates. We have previously discussed evidence (Stadie, Lukens, and Zapp, 1940) on the rate of formation of carbohydrate by diabetic livers, which fails to support this hypothesis. The data in Table X force us to the conclusion that it is untenable for

TABLE X

Non-Ketone Respiration of Liver Slices from Fasted Normal, Untreated Diabetic, and Insulin-Treated Diabetic Cats

200 mg. slices; 3.0 cc. of phosphate buffer; 2.0 hours; 38°.

Cat No.	O_2 uptake per gm. per 2 hrs.	Total R.Q.	Non-ketone O_2 per gm. per 2 hrs.	Total CO_2 per gm. per 2.0 hrs.	Non-ketone R.Q.
		Normal fasted cats			
	micromoles		*micromoles*	*micromoles*	
99A	139 ± 13	0.70 ± 0.02	118	97	0.82
99B	144 ± 0	0.67 ± 0.01	111	97	0.87
99C	151	0.49	137	74	0.72
Mean	142 ± 3	0.64 ± 0.03	122 ± 9	89 ± 9	0.81 ± 0.05
		Untreated diabetic cats			
79	157 ± 3	0.21	32	33	1.03
96A	192 ± 18	0.33 ± 0.04	21	64	3.04
96B	201	0.39	63	78	1.24
96C	163	0.40	25	65	2.60
96D	149	0.40	65	60	0.92
96E	177	0.22	44	39	0.89
Mean	185 ± 8	0.32 ± 0.04	42 ± 9	57 ± 8	1.36 ± 0.34
		Insulin-treated diabetic cats			
102B	167 ± 14	0.34 ± 0.03	126	57	0.45
102C	150 ± 6	0.51 ± 0.01	117	77	0.66
102D	152 ± 2	0.46 ± 0.04	117	70	0.60
Mean	156 ± 3	0.44 ± 0.06	120 ± 4	68 ± 7	0.57 ± 0.07

the following reasons. The long fatty acids (16 carbon atoms or more) occurring in the liver are initially essentially unoxidized. Therefore, for the conversion of the carbon atoms of the fatty acid into a hexose ($C_6H_{12}O_6$), *all* (*i.e.* 3) moles of oxygen per mole of hexose must be supplied ultimately by respiratory oxygen. Accordingly, there could be formed a maximum of carbohydrate per micromole of O_2, of $0.180/3 = 0.06$ mg. per micromole of O_2. The total oxygen available for carbohydrate formation from

TABLE XI

Proportion of Acetoacetate to Total Ketones Formed by Liver

Condition of cat	Cat No.	Acetoacetate, per cent of total ketones
Normal, fasted	99A	69
	99B	61
	99C	57
Mean		62 ± 4
Diabetic	79	32
	96A	40
	96C	51
	96D	65
	96E	45
Mean		44 ± 5
Insulin-treated diabetic	102A	42
	102B	56
	102C	40
	102D	46
Mean		46 ± 6

fat is the non-ketone oxygen. In our series of diabetic cat livers, the mean non-ketone oxygen was 21 micromoles per gm. per hour, or, since the average weight of the livers of the cats was 26 gm. per kilo, the approximate maximum possible carbohydrate formation from fatty acids was $0.06 \times 21 \times 26 = 33$ mg. per kilo of cat per hour. This is only a small fraction (16 per cent) of the total glucose (200 ± 30 mg. per kilo per hour) excreted by our diabetic cats in the preexperimental 1.0 hour period. But

our experiments show further that this small amount of non-ketone oxygen was entirely used up in the production of CO_2 (non-ketone R.Q. = 1.34 ± 0.34). In other words, there was no oxygen available in the metabolism of the diabetic liver slice for the oxidation of fatty acids to carbohydrate.

Proportion of Acetoacetate and β-Hydroxybutyrate to Total Ketone Formation by Liver—There are reasons for believing that the enzyme system concerned with the oxidation of fatty acids in the liver is adjusted so as to produce the two forms of ketones in fairly constant ratio to each other. This is brought out in Table XI. The total ketone formation in the diabetic cat is roughly divided between the two ketones in the ratio of 1:1. The higher value of acetoacetate in the normal cats is probably not significantly different from that of the diabetics.

We wish to express our thanks to Mildred S. Wright for her helpful criticisms and assistance in the experiments.

SUMMARY

1. With liver slices from fasted normal and diabetic cats, the rate of formation of ketones *in vitro* was determined. The urinary ketone excretion in a preliminary 1.0 hour period was also determined.

2. Ketone utilization by the muscles was calculated from measurements of (*a*) excess hepatic ketone formation over urinary ketone excretion, (*b*) oxidation of added acetoacetate by muscle mince equilibrated *in vitro*, (*c*) simultaneous equilibration of muscle mince with diabetic liver slices as source of ketones, (*d*) measurement of portal-hepatic blood ketone difference. An average value of 1300 micromoles per kilo of cat per hour was found for the utilization of ketones by muscle.

Utilization in the diabetic was found to be essentially the same as that in the normal cat. This demonstrates that the oxidation of ketones by muscle is independent of the presence of insulin.

3. No utilization of *dl*-β-hydroxybutyrate added to muscle mince could be demonstrated, either in normal or diabetic cats. This is a possible indication that acetoacetate is the sole form of ketone oxidized by muscle. There was found in four of six cases an increase both of β-hydroxybutyrate and total ketones, evidence

that muscle as well as liver may be a source of ketones in the diabetic.

4. The molecular ratio of oxygen consumed to ketone produced from fatty acids by the liver was found to be close to the value of 1.25 required by the multiple alternate oxidation hypothesis. This is evidence that the higher fatty acids yield not 1 but 4 molecules of ketones per molecule of fatty acid oxidized.

5. On the basis of the observed O_2 to ketone ratio, it is possible to calculate the *non-ketone* respiration of the liver and contrast its characteristics in the fasted normal, the diabetic, and the insulin-treated diabetic cat.

6. The non-ketone oxygen available for the possible oxidation of fatty acids to carbohydrates was found to be essentially zero. We conclude, therefore, that the hypothesis of the overproduction of carbohydrates from fats in diabetes is untenable.

7. Insulin in the presence of fructose, fumarate, and *d*-lactate inhibited the formation of ketones by the diabetic liver. This effect was enhanced by prolonged equilibration of slices with insulin-containing media.

8. Prior insulin treatment of diabetic cats restored the metabolism of the liver slice to a status intermediate between the normal and the diabetic. There was found a marked reduction of ketone formation and a type of non-ketone respiration suggesting partial oxidation of fatty acids beyond the ketone stage.

9. In Houssay cats we found that the ketone formation by the liver was essentially that of the fasted normal cat. This suggests that insulin controls ketone formation indirectly by acting antagonistically to the ketogenic pituitary hormone.

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THE PREPARATION OF A CONCENTRATED FECAL PHOSPHATASE AND ITS EFFECT ON DOGS AND RATS

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In a previous communication (1) it was found that the transfusion of phosphatase-rich, jaundiced blood into normal compatible recipients caused a marked and prolonged rise in the serum phosphatase level. This work suggested that some direct knowledge regarding the physiological significance of the enzyme might be obtained by maintaining an elevation of the serum phosphatase for short and prolonged periods of time. Little is known about the relation of this enzyme to metabolic processes, or its origin and rate of removal from the blood stream in normal and abnormal conditions. Since the transfusing of jaundiced blood is inconvenient and impracticable, it was decided to make a concentrated phosphatase preparation which could be administered parenterally and which would maintain the serum phosphatase activity at a high level.

EXPERIMENTAL

Preparation of Concentrated Fecal Phosphatase—A concentrated preparation of phosphatase was first prepared from kidney and intestinal mucosa according to the method of Albers and Albers (2). When these preparations were injected intravenously, it was found that the serum phosphatase returned to normal in the first half hour following its injection. A phosphatase preparation was then made from dog feces which Armstrong (3) has shown to be a potent source of this enzyme. The methods of Armstrong (3)

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and of Albers and Albers (2) were both used. It was found that the phosphatase from this source would persist in the serum for 48 hours or more after intravenous injection. However, such preparations were too toxic for our purposes. After various procedures were tried, the following method was adopted because it provided a less toxic and enzymatically more potent product than have various other procedures in our hands. Fresh (within 24 hours) dog feces were collected and mixed in a bucket with sufficient ice-cold tap water to form a sludge that could be poured easily. This extract must be kept cold, so it was customary to carry out the subsequent procedures in a refrigerator near 0°. The thoroughly mixed sludge is filtered through a large Berkefeld filter with the aid of suction and the filtrate should be a perfectly clear amber-colored liquid. 100 gm. of washed kaolin are added to each 500 cc. of fecal extract in a 2 liter flask. Sufficient glacial acetic acid is added to the kaolin-extract mixture to give a definite acid reaction with litmus paper. The flask containing the mixture is shaken vigorously for 5 minutes and allowed to stand until all of the kaolin has settled to the bottom. The supernatant fluid is siphoned off and the kaolin from two flasks is combined in one with the aid of small portions of ice-cold distilled water that has been acidified. The total volume is made to approximately 1500 cc. by the addition of more acidified ice water, and after a thorough mixing the kaolin is allowed to settle. The supernatant fluid is siphoned off and the washing repeated until the wash water remains clear and colorless. The subsequent procedure may be carried out at room temperature. The kaolin is then transferred to 50 cc. centrifuge tubes and the final water is removed by centrifugation. The packed kaolin is transferred to a 250 cc. beaker with the aid of 100 to 150 cc. of distilled water and the mixture is made slightly alkaline to litmus paper by the addition of ammonia water. After a thorough mixing the suspension is allowed to stand for 10 minutes, and is then filtered with the aid of suction; the first part of the filtrate usually needs to be returned for a second passage through the filter (Whatman No. 50). In this way a clear, light brown filtrate is obtained; it is neutralized to litmus with dilute acetic acid. Commercial dioxane is added to the filtrate to a concentration of 60 per cent by volume. The mixture is allowed to precipitate overnight and the supernatant

fluid is removed without disturbing the precipitate. The precipitate is transferred to a 50 cc. centrifuge tube and the remaining fluid decanted after centrifugation. The precipitate is washed with 100 per cent dioxane and recentrifuged; the air-dried, pulverized product thus obtained is a gray to brown powder. The potency of the enzyme obtained by this procedure varies from 10,000 to 19,000 Bodansky units for each gm. of powder. This powder has one optimum zone of pH for enzyme activity which is 9.0 to 9.8; its activity is increased by adding magnesium chloride to the buffer-substrate mixture. The powder is quite stable and shows no significant change in potency after being stored for several months.

Analytical Procedures—The following methods were used in analyzing serum and urine. All inorganic phosphorus and phosphatase determinations were made according to Bodansky's methods (4). Clark and Collip's method (5) was used for the serum calcium determination, while for the urine calcium determination, Wang's method (6) was adopted. The serum sugar was determined by the Shaffer-Hartmann-Somogyi method (7) and Somogyi's copper method (8) was used to prepare the protein-free filtrate from the serum for this determination.

Effect of Fecal Phosphatase Administered to Dogs—Dogs were used for a study of the immediate metabolic effect of this phosphatase preparation administered parenterally. The enzyme was dissolved (3 mg. per cc.) in 0.9 per cent saline that had been made slightly alkaline with a few drops of 5 per cent ammonia water, and injected intravenously at a dosage of 1.5 mg. per kilo of body weight. All dogs received the same powder, which was a mixture of several different preparations and which had a phosphatase activity of 17 units per mg.

The effects of an inactivated preparation were also studied. Inactivation was effected by adding sufficient acetic acid to the enzyme preparation to change its pH to 4.0 and by allowing the solution to stand at room temperature for 24 hours before the reaction was made slightly alkaline by the addition of ammonia. The dosage for the inactive was the same as for the active preparation.

Eighteen female dogs were studied under pentobarbital anesthesia. Ten of these dogs received the active and eight the

inactive preparation. Blood samples were drawn before and at several intervals after the injection of the preparation. Serum phosphatase, inorganic phosphorus, calcium, and sugar were determined on each sample. Urine was also collected at similar intervals by means of an indwelling, double current, bladder cannula and its inorganic phosphorus and calcium content determined.

TABLE I

Average Serum and Urine Values in Dogs before and after Injection with Active and Inactivated Enzyme Preparations

	Active preparation (10 dogs)					Inactivated preparation (8 dogs)			
	Before injection	5 min. after	1 hr. after	2 hrs. after	4 hrs. after	Before injection	1 hr. after	2 hrs. after	4 hrs. after
Phosphatase, *units* per 100 cc. serum* ...	2.73	33.11	17.15	15.10	13.00	3.17	3.38	3.25	3.32
Sugar, *mg. per 100 cc. serum*	116.00	124.00	116.00	106.00	89.00	97.00	106.00	112.00	101.00
Calcium, *mg. per 100 cc. serum*	11.10	11.40	10.80	10.80	10.90	11.20	11.40	11.20	11.00
Inorganic P, *mg. per 100 cc. serum*....	5.12		5.32	5.05	5.18	5.83	4.72	4.69	5.39
Urine inorganic P, *mg. per hr.*	15.38		14.54	9.07	5.54	15.05	15.53	7.82	8.83
Urine calcium, *mg. per hr.* .	0.51		0.79	0.96	0.37	0 74	0.86	0.69	1.23

* Bodansky units.

Table I gives the average results of serum and urine values in dogs injected intravenously with the enzyme preparation. After injection of the *active enzyme preparation*, the increased serum phosphatase disappeared from the circulation very rapidly during the 1st hour. Subsequently the fall was much more gradual and determination of the serum phosphatase 48 hours after injection still usually showed (Fig. 1) a higher level than that before injection of the active phosphatase preparation. Eight out of ten dogs showed a decrease in serum sugar of 20 mg. or more, the fall

occurring from 1 to 4 hours after the injection. Only two of the eight controls showed a similar decrease. A slight but insignificant decrease of serum calcium was indicated by the average figure. While the serum inorganic phosphorus was variable and the average figure did not show much change, the urine inorganic phosphorus showed a marked decrease after every injection. The

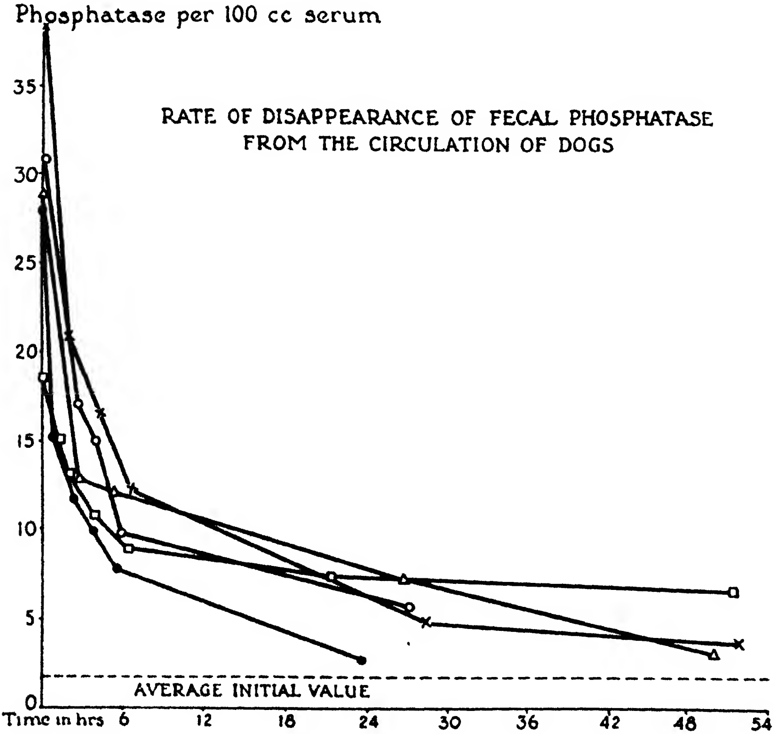

Fig. 1

change of urine calcium was irregular. In the dogs injected with *inactivated enzyme preparation*, no change of serum phosphatase occurred following the injection. A slight increase in serum sugar was found in most of the dogs. The change of serum calcium was about the same as that of the dogs receiving the active enzyme preparation. A fall of serum inorganic phosphorus occurred in the first 2 hours after the injection, but the fall of urine inorganic

phosphorus did not appear to be as marked as in the other group. The change of urine calcium was again variable.

Effect of Fecal Phosphatase Administered Intraperitoneally for 1 Month on Phosphatase Content of Serum, Kidney, and Tibia of Rats—The effect of this enzyme preparation on the tissue phosphatase content was studied on albino rats. Experiments began when the rats were 4 weeks old. The rats were divided into three groups. Group A received inactive and Group B active preparations, while Group C served as a control and was not injected. A daily dose of 1 mg. (19 units) of active or inactive preparation in 1 cc. of saline was given intraperitoneally throughout the experimental period, except for three rats in each group (Rats 1 to 3) whose daily dose was increased to 3 mg. after 2 weeks, and then, after 3 days, to 4 mg. for the remainder of the experiment. The injection period was a month, at the end of which time the rats were bled to death under ether anesthesia, and the left kidney and tibia were removed and freed from the surrounding tissues. These tissues were weighed and extracted for 24 hours with distilled water, to which 2 or 3 cc. of toluene were added, and their phosphatase content determined.

The results showing the effects of the enzyme preparation on the phosphatase content of serum, kidney, and tibia in the rats are given in Table II. They show that the phosphatase content of these tissues differs considerably from one rat to another, even in the control group, but the average values show that the serum phosphatase of those rats injected with the active preparation was higher than that of the other groups, while the serum phosphatase values of the control rats and of the rats injected with inactivated preparation were similar. The average values for bone and kidney phosphatase were similar in the two injected groups. Compared with the uninjected control rats, the kidney phosphatase of the injected groups was higher, while the reverse was the case for bone phosphatase.

Toxic Effects of Phosphatase Preparation—This phosphatase preparation at the dose used (1.5 mg. per kilo) was frequently found to produce toxic effects of varying degrees in unanesthetized dogs when injected intravenously, as manifested by defecation, vomiting, a fall in blood pressure, and weakness. A dose twice as large may be fatal to some dogs when given intravenously,

TABLE II

Phosphatase Content of Serum, Kidney, and Bone

	Rat No.	Sex	Weight	Serum		Kidney phosphatase	Tibia phosphatase
				Phosphatase	Inorganic P		
			gm.	*units per 100 cc.*	*mg. per 100 cc.*	*units per gm.*	*units per gm.*
Group C. Uninjected rats	1	F.	127	106 55	7.95	31.50	14.99
	2	M.	187				
	3	"	175	86.65	8.55	19.13	14.87
	4	F.	123				
	5	M.	183	63.53	8.75	28.12	14.45
	6	F.	125				
	7	M.	178	56.86	8.05	19.51	12.22
	8	"	196				
	9	"	170	67.24	8.05	13.71	10.50
	10	"	167				
Average			162.9	76.17	8.27	22.39	13.41
Group B. Injected with active phosphatase preparation	1	M.	153	159.03	9.00	30.21	13.26
	2	F.	121	107.15	7.90	44 32	13.72
	3	"	129	79.45	8 50	33.46	7.05
	4	M.	176	98.49	8.35	27.18	12.18
	5	F.	116				
	6	"	139	137.03	9.00	29.97	14.43
	7	M.	178				
	8	F.	127	71.12	7.50	22.74	10.20
	9	M.	204				
	10	"	163	97.34	7 30	26 00	10.19
	11	F.	139				
	12	M.	141	87 07	9.25	19.10	11.81
	13	"	140				
	14	"	116	97.63	8.15	19 19	12.09
	15	"	178				
Average			148	103.81	8.34	28.02	11 65
Group A. Injected with inactivated phosphatase preparation	1	M.	144	74.34	8.35	28.74	15.87
	2	F.	122	72.06	6.75	31.87	14.23
	3	"	134	46.98	7.60	44 91	6.61
	4	M.	168	87.56	9.15	30.54	11 75
	5	F.	138				
	6	"	114	64.62	8.15	29.22	7.82
	7	M.	178				
	8	F.	131	71.43	8.10	24.07	12.14
	9	M.	164				

TABLE II—*Concluded*

	Rat No.	Sex	Weight	Serum Phosphatase	Serum Inorganic P	Kidney phosphatase	Tibia phosphatase
			gm.	*units per 100 cc.*	*mg. per 100 cc.*	*units per gm.*	*units per gm.*
Group A—*Concluded*	10	M.	193	60.19	7.90	24.09	11.76
	11	F.	136				
	12	M.	134	105.39	8.25	19.85	12.35
	13	"	138	109.88	9 45	14.41	11.60
	14	"	156				
Average			141	76.94	8.19	27.52	11.57

but no or little reaction was observed after subcutaneous or intraperitoneal injection, even with large doses. Repeated intravenous injections seemed to result in a tolerance; repeated intraperitoneal injections with comparatively large doses did not cause symptoms in rats. The kidneys and livers of dogs and rats injected with active and inactive phosphatase preparation for varying lengths of time failed to demonstrate any gross or microscopic defects that could be related to the enzyme's activity. The final weight of the rats showed little difference between the two injected groups, but both of these groups have a lower average than that of the uninjected group.

DISCUSSION

In recent years, much speculation has been devoted to the relation of phosphatase to ossification, to the excretion of phosphates, to reabsorption of sugar from the renal tubules, to the absorption of carbohydrates and fats from the intestine, and to the formation of esters of phosphoric acid in the lactating mammary gland. The subject has been reviewed by Folley and Kay (9). Several experiments have tended to relate this enzyme to phosphorus, calcium, and carbohydrate metabolism. Bodansky (10) demonstrated an increase in serum phosphatase in dogs on ingestion of glucose or dextrin and a decrease in inorganic phosphorus when the blood sugar increased. The increase in serum phosphatase caused by a high carbohydrate-low protein

diet, or by pancreatectomy, suggests a relation between this enzyme and carbohydrate metabolism (11, 12). By administration of estrogenic hormone to lactating cows, Folley found a marked temporary rise in serum phosphatase which followed closely upon a drop in serum calcium and a change of serum inorganic phosphorus (13). Phlorhizin was reported to cause an increase in serum phosphatase and inorganic phosphorus (14). An increase in serum sugar with a decrease in serum inorganic phosphorus was found in dogs whose serum phosphatase was increased by transfusion with blood from animals with obstructive jaundice (1).

Armstrong (3) showed that dogs excrete large amounts of phosphatase daily in their feces, and described a method for preparation of a very active powder from this source. The potency which he reports is much higher than that which we obtained either by his method or the one described in this paper. However, the substrate used for assay was different and values obtained by the two methods cannot be compared. The origin of fecal phosphatase is unknown, but a few observations seem to indicate that as rats grow older they excrete less phosphatase in feces, and that relatively large injections of phosphatase fail to alter the fecal excretion of this enzyme by rats.

The change in the concentration of serum and urinary constituents observed after the injection of this enzyme preparation may be due to the enzyme itself or to some contaminant. The differences observed between the groups receiving the active and inactive preparations suggest that the enzyme itself may be of importance in such changes. Compensatory mechanisms on the part of the organism perhaps obscure the effect caused by the enzyme as a result of its synthetic or hydrolytic properties. The toxic manifestations are certainly not due to the activity of the enzyme, as the inactivated preparation has a similar effect. Further purification of the enzyme may simplify such studies by eliminating interfering substances. The curve showing the rate of disappearance of fecal phosphatase from the circulation is quite similar to that obtained when the serum of a jaundiced animal is transfused into a normal recipient (11), as in both instances the high levels, which may be predicted by calculation of the blood volume, are rapidly decreased until the concentration in the serum

approaches 10 units per 100 cc. of serum, after which the removal of the excess becomes gradual and may take days.

SUMMARY

A simple method is described for the preparation of phosphatase from dog feces. Such preparations were found to increase the serum phosphatase in dogs for at least 48 hours when injected intravenously. A study was made of serum calcium, inorganic phosphorus, and sugar in anesthetized animals injected with active and inactive preparations of this enzyme and of the urinary concentration of calcium and phosphorus in the same animals. No changes occurred in the serum or urine which could unquestionably be ascribed to the activity of this enzyme; however, a serum sugar decrease of 20 mg. or more occurred in eight out of ten anesthetized dogs receiving the active preparation, while only two out of eight dogs receiving the inactive preparation showed a similar decrease. An elevation of the serum phosphatase was maintained for 1 month in growing rats by the daily intraperitoneal injection of the enzyme preparation. No significant difference in the growth or well being could be noticed between groups receiving the active or inactivated preparations, although the group receiving the former preparation had a higher average serum phosphatase.

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8. Somogyi, M., *J. Biol. Chem.*, **90**, 725 (1931).
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LETTERS TO THE EDITORS

SULFONIUM REACTIONS OF METHIONINE AND THEIR POSSIBLE METABOLIC SIGNIFICANCE

Sirs:

Examples of sulfonium compounds of the composition $^{-}OOC{-}CH(NH_3^+)CH_2CH_2S(CH_3)R^+X^{-}$ may be obtained by letting methionine react with an excess of methyl bromide or methyl iodide in a 1:1 mixture of 85 per cent formic acid and acetic acid until titration of halide ion shows the reaction to have reached completion. Evaporation under reduced pressure and digestion of the residue with methanol yield the crystalline methyl sulfonium salts. They are water-soluble, neutral substances which give the theoretical values for halide ion. The analogous reactions with iodo- or bromoacetic acid occur readily in aqueous solution.

The capacity of methionine to form tertiary sulfonium ions—a reaction characteristic of organic monosulfides—may deserve attention from the metabolic standpoint. For the mechanism of the biological conversion of methionine to cystine[1] various possibilities have been discussed. These assume, generally with conversion of methionine to homocysteine, either transfer of the methionine sulfur to another carbon skeleton, by reactions involving addition to an unsaturated chain,[2] or a shortening of the methionine skeleton, for which the shift of a thiol group from the γ to β position, followed by ω oxidation and decarboxylation,[1] or an oxidative degradation at the aminocarboxylic end of the molecule[3] has been suggested.

Another working hypothesis can be based on the capacity of methionine to form sulfonium derivatives and the participation of hydroxyamino acids: $HOOC{-}CH(NH_2)CH_2CH_2SCH_3$ + HO-

$$OC{-}CH(NH_2)CH_2OH \rightarrow HOOC{-}CH(NH_2)CH_2CH_2{-}\underset{OH}{\overset{CH_3}{S}}{-}CH_2{-}$$

[1] Tarver, H., and Schmidt, C. L. A., *J. Biol. Chem.*, **130**, 67 (1939).

[2] Brand, E., Block, R. J., Kassell, B., and Cahill, G. F., *Proc. Soc. Exp. Biol. and Med.*, **35**, 501 (1936). Nicolet, B. H., *J. Washington Acad. Sc.*, **28**, 84 (1938).

[3] du Vigneaud, V., Dyer, H. M., and Kies, M. W., *J. Biol. Chem.*, **130**, 325 (1939).

$CH(NH_2)COOH$ → (by removal of CH_3OH and cleavage) $HS—CH_2CH(NH_2)COOH$. Similar reactions may be postulated for hydroxyglutamic acid or threonine, and combinations of decarboxylation and ω oxidation could lead to the same final product. According to this hypothesis the $—SCH_3$ group itself is an essential factor in the genesis of cystine from methionine. Recent evidence of Rose[4] and du Vigneaud[3, 5] shows clearly the importance of the methyl group in the metabolic utilization of homocysteine. If the latter is an intermediate in the biological conversion of methionine to cystine, that evidence may indicate that methyl groups, in the form of choline, etc., must be supplied only for the construction of the *methionine* required in protein synthesis, while the cystine requirements could still be covered by a direct degradation of homocysteine. If, however, it were to be shown that in a diet which contains merely enough methionine to satisfy the minimum *methionine* requirements,[6] choline, or other sources of available methyl, has to be supplied in order to make homocystine (or homocysteine) an effective substitute for cystine, a case would be established for the hypothesis here offered.

The biological occurrence of tertiary sulfonium compounds is suggested by the statement of Neuberg and Grosser[7] that normal dog urine contains methyldiethyl sulfonium ion. The biological importance of the analogous quarternary ammonium compounds is multiple (*e.g.* choline, thiamine) and the thiamine literature contains evidence[8] that living organisms possess enzymatic means to effect the reaction $R_3N + R'OH \rightarrow R_3R'N^+ + OH^-$. Therefore, the counterpart of this reaction in sulfur chemistry, postulated above, may also be within the scope of cell chemistry.

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GERRIT TOENNIES

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[4] Rose, W. C., and Rice, E. D., *J. Biol. Chem.*, **130**, 305 (1939).

[5] du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).

[6] Rose, W. C., *Science*, **86**, 298 (1937).

[7] Neuberg, C., and Grosser, *Centr. Physiol.*, **19**, 316 (1905).

[8] Abderhalden, E., and Abderhalden, R., *Arch. ges. Physiol.*, **240**, 746 (1938).

CONVERSION OF ETIOALLOCHOLAN-3,17-DIONE INTO ANDROSTERONE*

Sirs:

It has been previously demonstrated in men with evidence of deficient testicular secretion that the testis hormone, testosterone, may be converted into the urinary androgen, androsterone.[1,2] The mechanism of this conversion is obscure. It has been pointed out, however, that since androsterone has a particular spatial configuration at positions 3 and 5, that is $3(\alpha)$-hydroxy and 5-allo, there could be six direct intermediates between testosterone and androsterone.[2]

One of these possible intermediates, etioallocholan-3,17-dione,[3] has been fed in the amount of 100 mg. per day for 10 days to a man with evidence of deficient testicular secretion. All the urine was collected during this period. The titer of urinary androgens during treatment rose from a pretreatment level of 9.3 I.U. per day to 94 I.U. for a similar period. From the neutral fraction of the urinary extract, there were isolated 30 mg. of androsterone, m.p. 181–183° (uncorrected). The acetate melted at 163–165° (uncorrected). The melting points of neither the free compound nor the acetate were depressed when mixed with authentic samples. Thus it appears from these experiments that etioallocholan-3,17-dione may be an intermediate in the conversion of the testis hormone, testosterone, into the urinary androgen, androsterone.

* This work was aided by grants from the Rockefeller Foundation, the Fluid Research Fund of Yale University School of Medicine, and the International Cancer Research Foundation. The latter grant was made to Dr. James B. Hamilton of the Department of Anatomy.

[1] Callow, N. H., *Biochem. J.*, **33**, 559 (1939).

[2] Dorfman, R. I., Cook, J. W., and Hamilton, J. B., *J. Biol. Chem.*, **130**, 285 (1939).

[3] The etioallocholan-3,17-dione, m.p. 134° (uncorrected), was furnished by Ciba Pharmaceutical Products, Inc.

This and further work on the metabolism of testosterone will be described in detail in the near future.

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Received for publication, November 25, 1939

17-β-HYDROXYPROGESTERONE

Sirs:

In the course of work in this laboratory on the preparation of the various adrenal hormones in pure form from the glands of cattle we have encountered a new isomer of desoxycorticosterone. The compound crystallizes from ethanol or acetone in beautiful thin plates which melt[1] at 212–215°. The following analytical data were obtained: calculated for $C_{21}H_{30}O_3$, C 76.3, H 9.2, mol. wt. 330; found, C 76.0, 76.0, 76.0, 75.8, H 9.3, 9.1, 9.4, 9.2, mol. wt. 363 (Rast). Its specific rotation in chloroform is $[\alpha]_D^{27} = +102° \pm 3°$. On spectrographic examination it showed selective absorption in the ultraviolet with a maximum at 240 mμ characteristic of α, β-unsaturated ketones. The compound was recovered unchanged on treatment with acetic anhydride in pyridine at room temperature. It yields a disemicarbazone which darkens at 240°, sinters at 280–290°, and gradually blackens but does not melt below 360° (calculated for $C_{23}H_{36}O_3N_6$, C 62.1, H 8.1, N 18.9; found C 61.4, 61.3, H 8.1, 8.1, N 18.8). It yields a dioxime which sinters at about 240° and melts with decomposition at 250–251° (calculated for $C_{21}H_{32}O_3N_2$, N 7.8; found, N 7.9). On oxidation with chromic acid in glacial acetic acid at room temperature a neutral crystalline oxidation product was obtained which melted at 168–169° and showed no depression in melting point when mixed with an authentic sample of Δ^4-androstenedione-3,17, m.p. 167–168° (calculated for $C_{19}H_{26}O_2$, C 79.7, H 9.2; found C 79.2, H 9.6). From these findings it follows that the compound is a 17-hydroxyprogesterone. Ruzicka and Meldahl[2] report a melting point of 288° and $[\alpha]_D^{18} = +54°$ (dioxane) for the 17-α[3] compound.

[1] All melting points were taken in a Berl block and are uncorrected.

[2] Ruzicka, L., and Meldahl, H. F., *Helv. chim. acta*, **21**, 1760 (1938).

[3] For a discussion of the steric configuration of the adrenal steroids around carbon atom 17, see Reichstein and coworkers (Reichstein, T., and Gätzi, K., *Helv. chim. acta*, **21**, 1188 (1938); Reichstein, T., and Meystre, *Helv. chim. acta*, **22**, 728 (1939)).

This new compound therefore must be 17-β-hydroxyprogesterone. It elicits no progestational activity when tested at a 5 mg. dose level with a modified Clauberg technique. It has slight if any adrenal cortical activity but has androgenic activity of the same order as androsterone when tested in the castrate rat.

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Received for publication, November 27, 1939

A NEW ADRENAL BASE

Sirs:

We wish to report the isolation of a new phenolic base from the adrenal glands of cattle. It crystallizes from ethyl alcohol in colorless prismatic needles which melt[1] with decomposition at 219–221°. Analysis, calculated for $C_{12}H_{12}O_2N_2$, C 66.64, H 5.60, N 12.96; found, C 66.50, 66.62, H 5.47, 5.50, N 12.95. The compound gives a wine-red color with aqueous ferric chloride on warming and couples readily with diazotized *p*-nitraniline. With Millon's reagent it first gives a dirty green solution which on warming turns to a brown-red. A typical blue color is obtained with Gibbs' phenol reagent. It yields no pigment with potato tyrosinase. In alcoholic solution its ultraviolet absorption spectrum[2] exhibits three maxima at 231 mμ (ϵ = 15,000), 271 mμ (ϵ = 12,700), 300 mμ (ϵ = 11,000), with minima at 248 mμ (ϵ = 6800), 290 mμ (ϵ = 9800), and 360 mμ (ϵ = 0). It is not sufficiently soluble in camphor for a molecular weight determination by the micro-Rast procedure but a minimum molecular weight of 216 was fixed by analysis of an acetyl and a methyl derivative. Acetylation with acetic anhydride in pyridine on a steam bath yields an *o*-monoacetyl derivative, m.p. 176–177° (with decomposition). Analysis, calculated for $C_{14}H_{14}O_3N_2$, C 65.08, H 5.47, N 10.85, mol. wt. 258; found, C 64.95, H 4.97, N 10.96, mol. wt. (micro-Rast) 280, 293. There was evidence of slight decomposition of the acetate in camphor. That the acetate is an O and not an N derivative is indicated by the absence of a ferric chloride reaction and by the slow development of a blue color with 2, 6-dichloroquinone-chlorimide on standing in aqueous sodium bicarbonate. The phenolic base yields a monomethyl ether (m.p. 132–133° with decomposition) with diazomethane. Analysis, calculated for $C_{13}H_{14}O_2N_2$, C 67.79, H 6.13,

[1] All melting points were determined in a Berl block and are uncorrected.

[2] We wish to thank Professor D. T. Ewing of Michigan State College, East Lansing, for the spectrophotometric examination.

N 12.17; found, C 67.32, H 6.09, N 12.60. The base dissolves slowly in 0.1 N HCl to yield a pale yellow solution. The dihydrochloride is obtained by evaporating the solution to dryness and crystallizing from ethyl alcohol-ethyl acetate from which it separates as rosettes of needles (m.p. 215–216°, with decomposition; sinters at about 195°). Analysis (air-dried sample), calculated for $C_{12}H_{12}O_2N_2 \cdot 2HCl$, N 9.69; found, N 9.84. The salt apparently loses HCl on drying in a vacuum at 110°. After 2 hours the nitrogen content was 12.86 per cent; calculated for $C_{12}H_{12}O_2N_2$, N 12.96 per cent.

The new base was tested for its possible pressor and oxytocic activity by Mr. L. W. Rowe of this laboratory and found to be inactive. The compound is of interest, since, to our knowledge, it is the only phenolic amine other than adrenalin thus far isolated in pure form from the adrenal gland. We suggest the name *adrenodiamine*. Its further study may aid in our understanding of the biological synthesis and degradation of adrenalin.

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Received for publication, November 27, 1939

THE RELATION BETWEEN FACTOR U AND VITAMIN B_6

Sirs:

Several groups of investigators[1] have recently demonstrated the vitamin B_6 requirement of chicks. These findings have raised the question whether factor U and vitamin B_6 are identical. Factor U[2] is a water-soluble growth factor required by chicks receiving a diet consisting essentially of polished rice and washed fish meal and supplemented with riboflavin and the chick antidermatosis factor. It was previously suggested[2] on the basis of differences in distribution that the two factors were different, yellow corn being a relatively rich source of vitamin B_6 and a poor source of factor U. The experiments reported here with crystalline vitamin B_6[3] have shown that the basal diet employed for studying factor U is deficient in two factors, one of which is vitamin B_6.

The basal diet employed is composed of polished rice 67.5, water-washed fish meal 24, soy bean oil 3, sardine oil 0.5, salts 2.5, and acetone extract of whey 2.5 per cent (chick antidermatosis factor concentrate), thiamine 200 γ per cent, riboflavin 350 γ per cent, and hexane extract of alfalfa equivalent to 1 per cent of dried alfalfa. Chicks were depleted on this diet for 1 week and then placed on the experimental diets. Two separate experiments were made: (1) the growth-promoting effect of vitamin B_6 and yeast was compared; (2) a yeast filtrate and a yeast eluate were included

[1] Carter, C. W., and O'Brien, J. R., *Proc. 7th World's Poultry Cong. and Exposition*, 126 (1939). Hegsted, D. M., Oleson, J. J., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **130**, 423 (1939). Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, **42**, 180 (1939).

[2] Stokstad, E. L. R., and Manning, P. D. V., *J. Biol. Chem.*, **125**, 687 (1938).

[3] Supplied through the courtesy of Merck and Company, Inc., Rahway, New Jersey.

in addition to the yeast and vitamin B_6. The eluate was prepared from a yeast extract by adsorbing with fullers' earth at pH 2.0 and eluting with 50 per cent methanol containing dilute ammonium hydroxide. The supplements used and the results obtained in both experiments are shown in the table.

The results show that vitamin B_6 does not give the same growth response as yeast. Increasing the level of vitamin B_6 above 250 γ per cent produces no increase in growth. The greater part of the

Experiment No.	Supplement per 100 gm. diet	Weight, gm.			No. surviving
		7 days	21 days	28 days	
I. 6 chicks per group	None	60.1	82.0		3
	7.5 gm. yeast	61.6	176.0		6
	500 γ vitamin B_6	60.1	128.3		6
	1000 γ " "	59.8	126.5		6
	2000 γ " "	61.1	118.0		6
II.* 7 chicks per group	None	42.5	58.8	All dead	0
	7.5 gm. yeast	42.7	134.0	200	7
	250 γ vitamin B_6	42.5	98.9	130	6
	500 γ " "	42.2	97.7	119	6
	500 γ " " + yeast eluate ≎ 10 gm. yeast	42 4	112 0	173	6
	500 γ vitamin B_6 + yeast filtrate ≎ 10 gm. yeast	42.1	109.3	129	6

* The depletion diet used for the 1st week in Experiment II contained no chick antidermatosis factor supplement.

unknown factor is adsorbed on fullers' earth, the eluate showing some activity and the filtrate being entirely inactive.

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THE SPECIFICITY OF PROTEOLYTIC ENZYMES FROM TUMORS

Sirs:

It has been shown that the specificity of proteinases may be characterized by their action upon synthetic substrates of known structure and configuration.[1] This approach has now been applied to a study of the proteolytic enzymes in extracts of mouse sarcoma No. M-180, Bashford mouse carcinoma, Brown-Pearce rabbit carcinoma, a carcinoma of the human breast, and a sarcoma of human bone.[2]

Substrate	Source of enzymes; per cent hydrolysis				
	Bashford carcinoma, 0.70 mg. protein N per cc.	Breast carcinoma, 0.59 mg. protein N per cc.	Brown-Pearce carcinoma, 0.97 mg. protein N per cc.	Bone sarcoma, 0.66 mg. protein N per cc.	Mouse sarcoma, 0.22 mg. protein N per cc.
Benzoyl-*l*-arginineamide	58	68	59	51*	65†
l-Leucylglycylglycine	87	26	14	3*	61†
l-Leucineamide	20	0	10	25	71
d-Leucineamide	25	0	5	0	1
Carbobenzoxy-*l*-glutamyl-*l*-tyrosine	44	34	40†	52*	67†
Carbobenzoxy-*d*-glutamyl-*l*-tyrosine	18	36	0	0	

Cysteine was used as activator in all experiments. The values represent per cent hydrolysis in 24 hours (except when otherwise noted) at 40°, and have been corrected for the enzyme blanks. The pH of the experiments was 4.6 to 5.2.

* 6 hours.

† 4 hours.

[1] Fruton, J. S., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **6**, 50 (1938). Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **130**, 19 (1939).

[2] We are greatly indebted to Dr. A. Claude of the Rockefeller Institute for the mouse and rabbit tumors, and to Dr. C. P. Rhoads of the Memorial Hospital, New York, for the human material.

It will be noted from the accompanying table that the carcinoma and bone sarcoma extracts split *l*-leucineamide either not at all or rather slowly. The Bashford carcinoma extract hydrolyzed *l*- and *d*-leucineamide at similar rates. All the tumor extracts hydrolyzed carbobenzoxy-*l*-glutamyl-*l*-tyrosine. The breast and Bashford carcinomas acted also on carbobenzoxy-*d*-glutamyl-*l*-tyrosine which contains the unnatural *d*-glutamic acid.[3]

The enzyme solutions obtained from carcinomas are much less stable than are those of the other tissue extracts, and they also show a marked autodigestion at pH 4 to 5 in the presence of cysteine.

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George W. Irving, Jr.
Max Bergmann

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[3] Kögl and collaborators (*Z. physiol. Chem.*, **258**, 57 (1939); **261**, 154 (1939)) have reported the isolation of *d*-amino acids from hydrolyzed tumors and have advanced a stereochemical theory of cancer based on these isolations. Their experiments are at present the subject of controversy in the literature.

COMPARISON OF THE ANTIHEMORRHAGIC ACTIVITY OF NATURAL AND SYNTHETIC VITAMIN K_1 WITH THE PROPOSED STANDARD 2-METHYL-1,4-NAPHTHOQUINONE

Sirs:

Owing to the various bioassay methods used, it is becoming more and more difficult to compare the vitamin K potency values of preparations from different laboratories.[1] To overcome this we have tentatively adopted the suggestion made jointly from our laboratory and that of Doisy:[2] the use of 2-methyl-1,4-naphthoquinone as a standard of reference. Ansbacher and Fernholz[3] found it exceptionally potent and, since it is stable and easily handled, it can well serve the purpose. By defining the biological activity of 1 γ of this quinone as 1 standard unit of vitamin K_1, we find in a series of six experiments with some 260 chicks, using the Thayer-Doisy method[4] (running the standard concurrently and using the master curve), that it is equivalent to 1.98 of our curative chick units. This makes our conversion factor 0.5.

In accordance with this plan, we report for comparative purposes the potency of samples of both the natural and synthetic vitamin K_1 in our units and the proposed ones. The samples were supplied by Dr. Doisy and by Dr. Major of Merck and Company, Inc.

It is apparent from the data that natural and synthetic vitamin K_1 have substantially the same values, and that the average potency (450) is very nearly 500 standard units per mg. This

[1] Snell, A. M., and Butt, H. R., *J. Am. Med. Assn.*, **113**, 2056 (1939).

[2] Thayer, S. A., Binkley, S. B., MacCorquodale, D. W., Doisy, E. A., Emmett, A. D., Brown, R. A., and Bird, O. D., *J. Am. Chem. Soc.*, **61**, 2563 (1939).

[3] Ansbacher, S., and Fernholz, E., *J. Am. Chem. Soc.*, **61**, 1924 (1939).

[4] Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., *Proc. Soc. Exp. Biol. and Med.*, **40**, 478 (1939).

Vitamin K_1	Sample No.	Dose level	No. of chicks	Per cent clotting under 10 min.	Average clotting time	Chick units per mg.	Potency based on 2-methyl-1,4-naphthoquinone
		γ			min.		units per mg.
Natural	Doisy, 30479	0.56	15	27	15.7		
		0.77	15	47	14.0	809	405
		1.11	14	79	7.6		
	" 31089	0.5	14	7	22.7		
		1.0	14	50	11.9	1020	510
		1.5	15	80	7.9		
	Merck, 39RD1546	0.6	13	15	19.0		
		1.2	15	40	15.3	690	345
		1.8	15	60	10.1		
Synthetic	Doisy, 30489	0.56	13	46	12.0	1076	535
		0.77	13	70	9.4		
	" 31079	0.5	15	13	17.2		
		1.0	13	62	9.8	1160	580
		1.5	13	86	6.6		
	Merck, 39RD1670	0.6	14	36	20.3		
		1.2	15	53	11.5	1020	510
		1.8	13	85	9.2		
	" 39RD1670	0.6	9	11	17.6		
		1.2	10	70	8.3	945	473
		1.8	10	80	7.0		
	" 39RD1821	1.2	15	47	7.4	808	404
		1.8	13	85	6.1		
	" 39RD1821	1.2	15	40	7.5	703	357
		1.8	15	80	6.2		
Average, natural vitamin K_1						840	420
" synthetic vitamin K_1						960	480

gives a somewhat higher value for natural vitamin K_1 than the 262 units reported recently by Almquist and Klose.[5]

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[5] Almquist, H. J., and Klose, A. A., *J. Biol. Chem.*, **130**, 787 (1939).

SYNTHESIS OF VITAMIN K_1

Sirs:

It seems desirable to describe in more detail the method for the synthesis of 2-methyl-3-phytyl-1,4-naphthoquinone (vitamin K_1) which we have announced elsewhere.[1] To 1 gm. of 2-methyl-1,4-naphthoquinone were added 2 cc. of petroleum ether, 1 gm. of zinc dust, and 0.6 cc. of phytyl bromide prepared from phytol[2] by the action of phosphorus tribromide. The mixture was refluxed in the dark for 20 hours. The reaction product from the reduction and condensation was obtained in hexane solution, washed repeatedly with water, filtered, and fractionated in a molecular still of a type previously described[3] but much larger. Unchanged 2-methyl-1,4-naphthoquinone and phytyl bromide were removed in the first distillate fraction. The second or vitamin fraction was redistilled. It was then placed in methanol and crystallized out repeatedly by cooling with solid carbon dioxide[3] and centrifuging, the filtrate being discarded each time. The product we obtained was practically pure 2-methyl-3-phytyl-1,4-naphthoquinone. Analyses for carbon and hydrogen gave C 82.1, 82.5, H 10.7, 10.6; calculated for $C_{31}H_{46}O_2$, C 82.6, H 10.3.

The potency of this preparation was approximately 56,000 cc. of our reference standard per gm. by chick assay.[4] A natural preparation of vitamin K_1[5] assayed concurrently gave a value of 61,500. This value agrees closely with others we have published.[6] An assay of a sample of synthetic vitamin K_1[5] prepared by a different process has yielded a value of 57,000. The in-

[1] Almquist, H. J., and Klose, A. A., *J. Am. Chem. Soc.*, **61**, 2557 (1939).

[2] We are greatly indebted to Professor Gordon Mackinney for highly purified chlorophyll from which we obtained phytol.

[3] Almquist, H. J., *J. Biol. Chem.*, **120**, 635 (1937).

[4] Almquist, H. J., and Klose, A. A., *Biochem. J.*, **33**, 1055 (1939).

[5] Kindly provided by Professor E. A. Doisy.

[6] Almquist, H. J., and Klose, A. A., *J. Biol. Chem.*, **130**, 787 (1939).

tensity of the color reaction[7] of these synthetic specimens was the same as that of the vitamin from natural sources. It is probable that the synthetic product contains a small amount of cyclized vitamin K_1.

Our work has been greatly aided by a grant from Merck and Company, Inc.

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[7] Almquist, H. J., and Klose, A. A., *J. Am. Chem. Soc.*, **61**, 1610 (1939).

THE SPLITTING OF DIGITONIDES

Sirs:

The only practical method for the recovery of steroids and digitonin from digitonides is the one recommended by Schoenheimer.[1] While this method gives satisfactory results on small quantities of digitonides, it is rather inconvenient when applied to the splitting of larger amounts. The filtration of considerable volumes of an ether-pyridine mixture containing a suspension of digitonin as well as the recovery of the digitonin is time-consuming.

These inconveniences can readily be overcome by the following simple modification of Schoenheimer's method.

The digitonide is dissolved in from 10 to 20 times its weight of dry pyridine and the solution is kept at 70-100° for about 1 hour. The pyridine is then removed as completely as possible by distillation *in vacuo*. The residue is treated twice with anhydrous ether, after which it is ground to a fine powder and extracted with anhydrous ether in a Soxhlet apparatus for 1 hour. The ether extracts are combined, filtered, and evaporated to dryness. The residue is then dried to constant weight. It represents the steroid which is obtained in yields of over 90 per cent.

The digitonin which remains after the ether extraction is also obtained in excellent yield. It is treated with sufficient hot 90 per cent alcohol to make a 1 per cent solution and the solution is filtered after 24 hours standing. The undissolved digitonide is then subjected to a second treatment with pyridine. Further quantities of steroid are thereby obtained which bring the total yield of recovered steroid to 95 to 98 per cent.

Example—4.5757 gm. of cholesterol digitonide gave 1.0716 gm. of cholesterol or 93.6 per cent of the theory after the first treatment with pyridine. 3.4820 gm. of the recovered digitonin contained 0.2620 gm. of material insoluble in cold 90 per cent alcohol. After treatment with pyridine it gave 0.0502 gm. of

[1] Schoenheimer, R., and Dam, H., *Z. physiol. Chem.*, **215**, 59 (1933).

cholesterol. Hence the total yield of recovered cholesterol was 1.1218 gm. or 98 per cent of the theory.

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A BIOCHEMICAL STUDY OF THE FERMENTATION OF RARE SUGARS BY MEMBERS OF THE COLON AND AEROGENES GROUPS OF BACTERIA

III. *d*-FUCOSE

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Most of the quantitative work on the fermentation of sugars and higher alcohols has been done on the more common members of these groups of compounds. An investigation has been started in this laboratory on the metabolic products which are formed by members of the colon and *aerogenes* groups of bacteria in media containing some of the rarer sugars. The results of the work with trehalose (1) and cellobiose (2) have already been reported. The investigation covered in this communication gives the results when *d*-fucose was used as the carbohydrate constituent of the medium.

The methods and procedures were essentially those employed in previous studies (1–3). The cultures used for the fermentations were members of the *Escherichia* and *Aerobacter* groups of bacteria.

For the quantitative determination of the products of fermentation, a medium was used containing 5 gm. of fucose and 8 gm. of Bacto-Nutrient broth in 1 liter of water. This medium was adjusted to a pH value of 7, and 100 cc. amounts were placed in Smith's fermentation tubes and 10 cc. amounts in Durham tubes and both were properly sterilized. Each of the Smith tubes was previously fitted at the top of the gas arm with a small bore glass tube, which was sealed with a short piece of rubber tubing containing a plug of solid glass rod. This arrangement was made in order to facilitate the transfer of the gas from the Smith tubes to the burette used for the analysis of the gas.

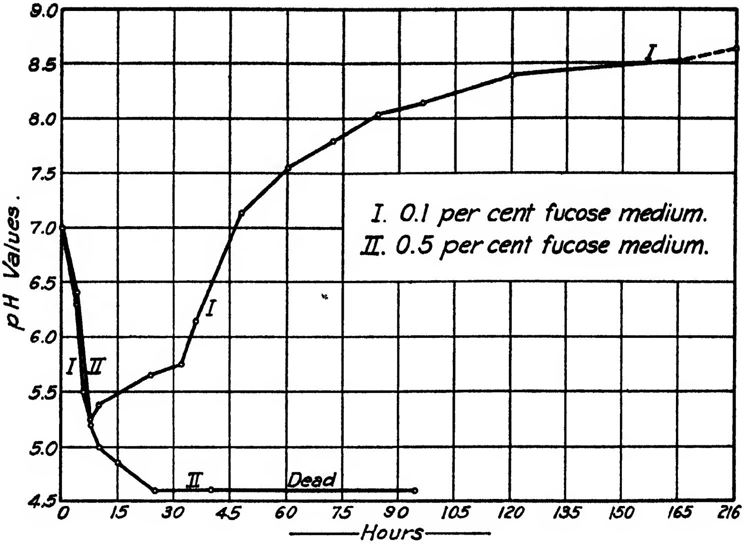

FIG. 1. Curves showing the change in pH values when *Escherichia coli* is grown in *d*-fucose media. The broken lines represent a different interval of time.

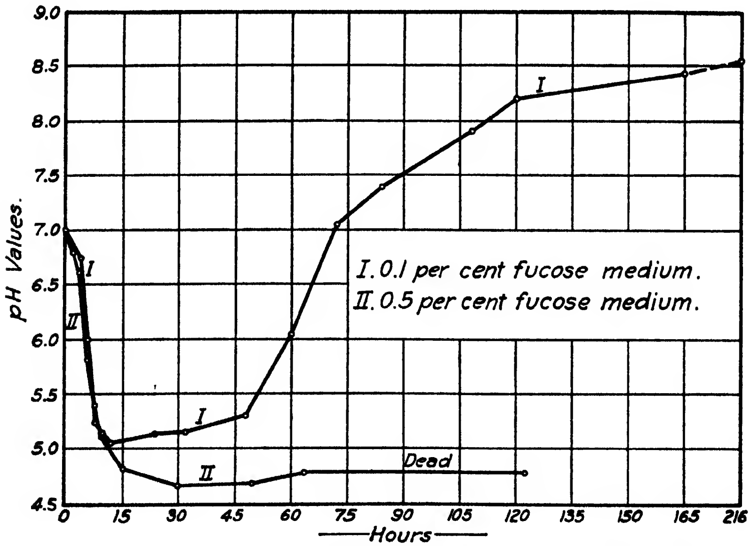

FIG. 2. Curves showing the change in pH values when *Aerobacter aerogenes* is grown is *d*-fucose media. The broken lines represent a different interval of time.

Some forty-two organisms were inoculated into medium contained in Durham fermentation tubes, in order to determine the amounts of gas formed during different periods of incubation at 37°. The production of gas took place very slowly. In 96 hours the colon cultures averaged 8 per cent gas and the *aerogenes* cultures 11 per cent gas. Rough determinations of the pH values were made in these same tubes every 24 hours up to 144 hours.

TABLE I

Fermentation Products in Fucose Medium

	Colon group				*Aerogenes* group			
	Escherichia coli	*Escherichia formica*	*Escherichia communior*	Average	*Aerobacter aerogenes*	*Aerobacter cloacæ*	*Aerobacter levans*	Average
	Culture 37	Culture 8	Culture 3		Culture 123	Culture 5	Culture 161	
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
CO_2*	21	12	13	15.3	15 5	12	13	13.5
H_2*	45	28	29	34.0	32 5	22	26	26.8
Volatile acids†	13.9	14.4	14.2	14.2	17.7	18.8	18.2	18.2
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Formic acid	1‡	Trace	1	0.7	2	1	2	1.7
Acetic "	82	86	84	84.0	103.6	111.5	106.6	107.2
Succinic "	10	10	9	9.7	37	42	31	36.7
Lactic "	15	9	11	11.7	24	20	16	20.0
$\frac{\text{Volatile}}{\text{Non-volatile}}$	3.32	4.52	4.25	4.03	1.73	1.82	2.31	1.95
$\frac{\text{Acetic}}{\text{Succinic}}$	8.2	8.6	9.33	8.71	2.80	2.66	3.44	2.97
$\frac{\text{Acetic}}{\text{Lactic}}$	5.47	9.56	7.64	7.56	4.32	5.58	6.67	5.52
Alcohol	26	206	390	207	210	215	108	178

* Volume in cc. formed in a Smith fermentation tube.

† Amount of 0.1 N alkali used to neutralize the acid from 1 gm. of sugar.

‡ All of the values in mg. are on the basis of 1 gm. of fucose.

Determinations of pH values were also made at more frequent intervals with the hydrogen electrode potentiometer in media containing 0.1, 0.5, and 1.0 per cent fucose. Various strains of four organisms were used, *Aerobacter aerogenes*, *Escherichia coli*, *Escherichia formica*, and *Escherichia communior*. The values

for the last two organisms were practically identical with those for *Escherichia coli*. Also the values obtained with the medium containing 1 per cent sugar were about the same as those with the medium containing 0.5 per cent sugar. The average values for three strains of *Escherichia coli* and those for three strains of *Aerobacter aerogenes* are given in Figs. 1 and 2.

The pH values with both groups of organisms in the medium containing 0.1 per cent sugar reached a minimum in about 7 hours, and then the value gradually increased until a maximum of about 8.7 was reached. The minimum was considerably lower in the medium containing 0.5 and 1.0 per cent sugar; the acidity which developed, however, killed the organisms in about 30 hours.

The media contained in the flasks and the Smith fermentation tubes were inoculated and incubated for 72 hours at 37°. The fermentation products were analyzed, and the results noted in Table I.

The amounts of the products formed with the two groups of bacteria showed considerable difference. The *aerogenes* group, on the whole, showed larger amounts of each of the constituents except the gases and alcohol than did the colon group. The *aerogenes* group produces on the average twice as much lactic acid and 4 times as much succinic acid as does the colon group. Another significant difference is found in the acid ratios. The ratio for the volatile to non-volatile acids and the ratio for the acetic to succinic acid were in each instance more than twice as large for the colon group. All the members of the *aerogenes* group gave tests for acetylmethylcarbinol, whereas those of the colon group did not.

SUMMARY

1. The fermentation products by members of the *Escherichia* and *Aerobacter* groups of bacteria in *d*-fucose medium have been determined.

2. The progressive changes in pH values during fermentation have been studied.

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THE EFFECT OF HOT ALCOHOL ON PURIFIED ANIMAL PROTEINS*

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(Received for publication, September 25, 1939)

The effect of heat or alcohol extraction on the nutritive value of proteins continues to be an interesting problem. Most of the current work on it has been summarized in several recent papers (1–3). Heat treatment usually lowers the nutritive value, although soy bean protein seems to be modified in the opposite direction (4). In long time feeding experiments the possibility of thermal destruction of unidentified accessories had to be reckoned with but short time balance experiments have left no doubt that the protein itself is changed in a manner such that either it is less digestible or its amino acid fragments after digestion are less utilizable than is the case with the native protein. The latter view is the more generally accepted, although no chemical evidence has been offered which would indicate any alteration or destruction, especially of lysine or histidine, the amino acids held to be the first limiting nutritional factors in heat-treated proteins. Indeed, Block, Jones, and Gersdorff (5) found no difference between the lysine content of heated casein and that of the unheated.

The work to be reported here had its origin in the observations of Seegers and Mattill (6) that beef liver, carefully dried at temperatures below 60°, suffered considerable loss of its value as a source of protein for growth and maintenance by being heated at 120° for 72 hours or by extraction with 95 per cent alcohol for 130 hours at the boiling point. When the two treated proteins and the untreated were hydrolyzed by acid, the resulting amino

* From a thesis presented by Robert L. Harris to the Faculty of the Graduate College of the State University of Iowa in partial fulfilment of the requirements for the degree of Master of Science.

acid mixtures were about alike in nutritive value. Digestion *in vitro* indicated that the treated liver was somewhat resistant to tryptic digestion. From these observations it was concluded that as a result of heating or alcohol extraction certain new linkages were formed which were not amenable to the action of proteases.

Muscle tissue when subjected to heat treatment suffered no deterioration at 110° but was altered by heating above 120° for 3 days (7), whereas kidney tissue showed no change on being heated at 130° but did so above that temperature. This indicated a rather fundamental difference in the composition of the proteins of these tissues or in the nature of the associated substances, manifesting itself in variable sensitiveness to this kind of denaturation.

It was therefore decided to fractionate the proteins of liver and kidney by the usual empirical solubility methods: to subject a portion to prolonged alcohol extraction, and to compare the extracted and unextracted proteins as to differences between them, on the basis of chemical criteria and by more extensive digestion experiments *in vitro*.

EXPERIMENTAL

For purposes of fractionation the method devised by Luck (8) seemed suitable for separating the proteins of fresh slaughterhouse tissues according to their solubilities in 5 per cent sodium chloride, 0.25 per cent sodium hydroxide, 3 M sodium sulfate, and water. To obtain consistent results it was necessary to regulate carefully the concentration of salts, temperature, time of extraction, and pH.

The quantities of the different proteins isolated (Table I) fall within the limits found by Luck, with the exception of the pseudoglobulin fraction, of which none appeared. Our material was not freshly excised; the tissue had been removed from slaughterhouse animals several hours before; nor was it frozen with liquid air before grinding, as Luck suggests.

The distribution between globulin II and euglobulin depends on slight changes in concentration of alkali or acid, on variations as to length of time allowed for centrifugation, and many other factors. The amounts of euglobulin isolated from kidney and liver are quite different, but the meaning of this difference is

obscure. The sum of the globulin and euglobulin in each case is about the same and the distinction between the two may not be sharp.

For extraction with 95 per cent alcohol, a portion of the protein was placed in the usual parchment thimble in a Soxhlet extractor and the extraction was continued for 5 days. Both extracted and unextracted proteins were then subjected to digestion *in vitro* by pancreatin.

1 gm. of the protein, containing 146 mg. of nitrogen, was placed in a 125 cc. flask, with 50 cc. of 0.3 per cent sodium carbonate solution containing 0.1 per cent sodium fluoride, together with 5 cc. of 3 per cent solution of commercial pancreatin (Wilson). The

TABLE I

Proteins of Beef Liver and Kidney

The figures on beef tissues are averages of at least five determinations.

Fraction	Beef liver	Beef kidney	Rat liver (Luck (8))	
			High protein	Low protein
	gm. per 100 gm.	*gm. per 100 gm.*	*gm. per 100 gm.*	*gm. per 100 gm.*
Globulin II	7.94	6.17	7.08–10.03	5.66–9.27
Euglobulin	2.76	4.85	4.66– 7.59	2.64–4.45
Albumin	1.83	2.15	1.86– 2.50	1.16–2.06
Residue	1.37	3.59		
Pseudoglobulin			1.63– 1.78	1.16–1.56

digestion mixture was covered with toluene and placed in a constant temperature room at 37°. The blanks were prepared in the same way except that the enzyme was added at the end of the digestion period. After 24 to 48 hours, the mixture was brought to boiling and precipitated with trichloroacetic acid. The filtrates were made up to 100 cc., total nitrogen was determined by the Kjeldahl method, and amino nitrogen by the Van Slyke micromethod.

From the figures in Table II it appears that the globulin II fraction of liver was the only one of the proteins to show any marked decrease in digestibility as a result of alcohol extraction. Attention was therefore concentrated upon this and the same fraction from kidney tissue.

The progress of digestion of the globulin II from liver and kidney is shown in Fig. 1. The difference in behavior between the liver and kidney globulin after extraction is obvious. From the very beginning, the digestion of the extracted liver globulin II was retarded; a higher concentration of enzyme accelerated the digestion, but the final equilibrium was not changed.

Other organic solvents had a similar effect upon the digestibility of liver globulin II. Portions of the extracted protein and of the original material were digested with pancreatin in the manner

TABLE II

Effect of Alcohol Extraction on Digestibility of Beef Liver and Kidney Proteins (48 Hours in Vitro)

The original substrates contained 146 mg. of protein nitrogen; the percentages are based on this figure and are the averages of at least five separate experiments.

		Amino N		Trichloroacetic acid-soluble N	
		Untreated	Alcohol-extracted	Untreated	Alcohol-extracted
		per cent	*per cent*	*per cent*	*per cent*
Liver	Globulin II	26.0	16.7	97.2	73.0
	Euglobulin	22.4	19.5	95.5	83.6
	Albumin	18.9	17.8	80.0	78.2
	Residue	14.4	14.1	66.6	66.5
Kidney	Globulin II	25.0	23.8	97.5	89.9
	Euglobulin	24.1	22.2	96.0	89.5
	Albumin	18.6	17.5	78.3	78.1
	Residue	14.0	13.7	65.0	65.0

previously described and precipitated with trichloroacetic acid, and the filtrates were made up to 100 cc. and analyzed as before. The results of these determinations (Table III) show that each of these extractants lowered the digestibility. Primary alcohols of low molecular weight caused a greater change than the corresponding secondary alcohols. Ketones had about the same effect as secondary alcohols, and dry heat at 110–120° for the same length of time produced the most drastic change. All of the solvents are dehydrating agents.

During these extractions hydrogen sulfide was evolved. For quantitative confirmation of the loss of sulfur the globulin II was

hydrolyzed by acid before and after alcohol extraction and in the hydrolysate cystine and cysteine were determined by the

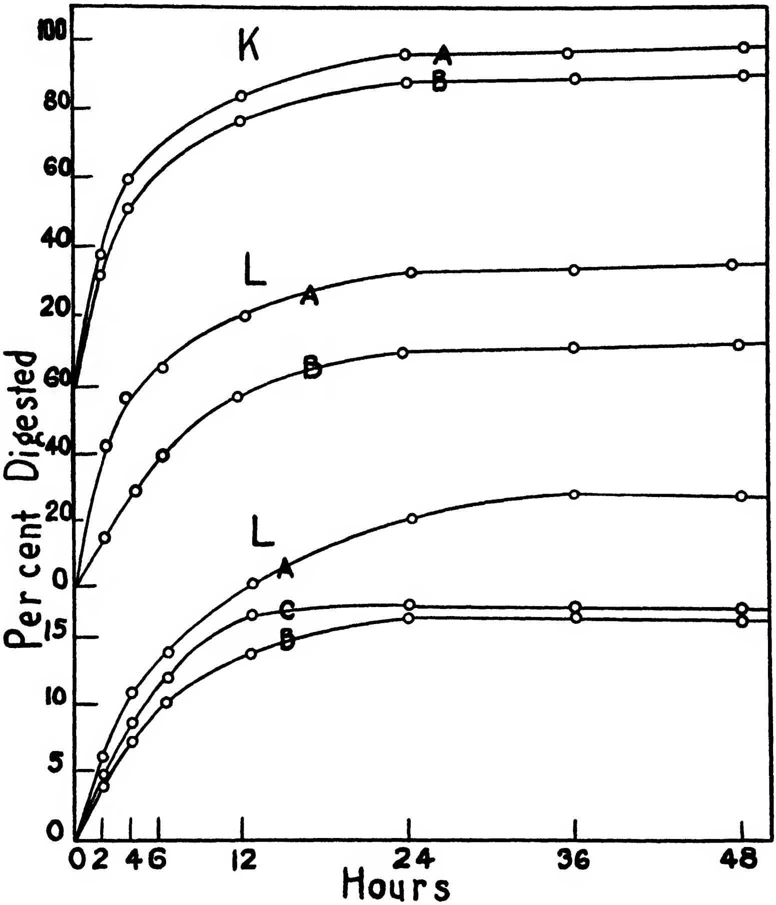

Fig. 1. Pancreatic digestion of liver (*L*) and kidney (*K*) globulins before (Curve A) and after (Curve B) extraction with hot alcohol. In *L*, Curve C, the concentration of enzyme was doubled. The three lower curves indicate increase in amino nitrogen; the four upper curves show increase in trichloroacetic acid-soluble nitrogen.

method of Shinohara (9), total sulfur by the Parr bomb. During extraction the total sulfur decreased from 12.9 to 7.7 mg. per gm.,

cystine sulfur from 11.6 to 5.71 mg. per gm. When cystine and cysteine were similarly extracted, hydrogen sulfide was evolved.

To reveal other possible changes in their amino acid content, the nitrogen distribution in the extracted and unextracted proteins was determined by the method of Van Slyke (10) as modified by

TABLE III

Digestion in Vitro of Liver Globulin II after Extraction with Various Organic Solvents

The percentages are based on 146 mg. of protein nitrogen which the substrates contained.

Extractant	Amino N in digest (Sørensen)	$CCl_3 \cdot COOH$ filtrate	
		Amino N (Van Slyke)	Total N
	per cent	*per cent*	*per cent*
Untreated	27.0	26.4	97.3
Ethyl alcohol (95%)	18.7	17.0	70.9
" " (100%)	18.9	16.6	71.4
Methyl "	18.1	16.3	70.9
Acetone (2 days)	21.2	20.0	79.5
" (5 ")	18.5	17.9	74.3
n-Propyl alcohol	18.6	17.6	74.6
Isopropyl alcohol	20.2	18.0	73.5
Dry heat, 120°	15.6	15.5	68.3

TABLE IV

Free NH_2 Nitrogen in Liver and Kidney Globulins

		Van Slyke	Sørensen
		mg. per gm.	*mg. per gm.*
Liver globulin	Alcohol-extracted	3.2	3.4
	Unextracted	7.0	7.4
Kidney globulin	Alcohol-extracted	5.9	1.4
	Unextracted	9.2	4.4

Cavett (11). Except for cystine there was no appreciable change; in particular, histidine and lysine were unchanged nor was there any marked difference in the distribution of amino acids in liver and kidney globulin (data available but omitted here).

Chemical evidence of some fundamental change produced by hot alcohol extraction was finally obtained when the free amino

nitrogen of the unextracted and extracted proteins was determined (by Van Slyke's amino nitrogen method and Sørensen's formol titration). For these determinations proteins were finely ground and suspended in distilled water. The values by both methods were checked at different periods with varying amounts of material (200 to 500 mg.). The results (Table IV) show that hot alcohol extraction reduced the amount of free amino nitrogen in both kidney and liver globulins. The methods are admittedly inadequate when applied to solid material. Complete solution of the proteins requires considerable amounts of alkali in whose presence the methods cannot be applied. The wide discrepancy between the Van Slyke and Sørensen figures on kidney globulin is particularly unsatisfactory.

DISCUSSION

Since hot alcohol extraction produced no loss of nitrogen from the proteins, the decrease in amino nitrogen can only indicate the disappearance of these free amino groups into new linkages. If the free amino groups in proteins are for the most part in lysine, slightly more than half of the ϵ-amino groups of lysine disappear during alcohol extraction. What new bonds are thus established can only be surmised, but under conditions favorable to dehydration the ϵ-amino groups of lysine may form anhydride linkages with available carboxyl groups to form compounds like the indigestible diketopiperazines (12). Imino groups and hydroxyamino acids offer similar possibilities. It has been shown (2, 1) that the addition of small amounts of histidine and of lysine (0.2 per cent) improves the nutritive value of diets containing heat-treated protein. The evidence from nitrogen partition and the actual isolation of like amounts of lysine by hydrolysis of heated and unheated proteins (5) indicate that lysine is not destroyed. The data on nitrogen partition also lend no support to the claim (13) that denaturation converts diamino acid nitrogen into other forms. One must also conclude that, if new linkages are formed by dehydration, those produced in kidney globulin are still accessible to the action of pancreatin, whereas those in liver globulin are not. Further studies, by other methods, are needed to explain this difference.

The possible participation of unessential amino acids in the

formation of anhydride complexes would obviously not be revealed by nitrogen balance studies. The fact that cystine and cysteine are decomposed and some organic sulfur is lost is also inconsequential from the nutritional point of view. An altered configuration even of soluble and absorbable fragments might make them unavailable to the animal because the specificity required for resynthesis was lacking. Denaturation of this kind might thus reduce both "biological value" and digestibility without damaging any essential amino acid. Recently (14) soy bean protein has apparently furnished an example of the converse process. Soy beans contain a complex of S and N which is absorbable but which cannot be used for tissue-building purposes. Heating the soy beans makes it available.

The authors are indebted to Dr. J. I. Routh for his helpful suggestions, especially with reference to the sulfur-containing amino acids, and to Mr. R. L. Borchers for some amino acid determinations.

SUMMARY

Liver and kidney proteins of freshly slaughtered beef were fractionated according to the method of Luck; the quantitative distribution of the different fractions of liver was similar to that found by Luck in freshly excised rat liver.

Of these protein fractions liver globulin II was the only component to show a marked decrease in digestibility *in vitro* by pancreatin after hot alcohol extraction. A similar result followed extraction with many other dehydrating organic solvents.

Similar nitrogen distribution was found in liver globulin II and kidney globulin II. Hot alcohol extraction of liver globulin did not alter this, except for cystine; both kidney and liver globulins lost much of their sulfur as hydrogen sulfide. The same occurred with cystine and cysteine.

The amount of free amino nitrogen in liver and kidney globulins was greatly decreased (50 per cent) by hot alcohol extraction. Since there was no loss in total nitrogen, this decrease may be due to the formation of new anhydride linkages involving the ϵ-amino group of lysine and available hydroxyl groups.

A lowered nutritive value of heat- or alcohol-treated proteins

may thus be caused primarily by the formation of new enzyme-resistant configurations involving essential amino acids. The bearing of this on the "biological value" of such denatured proteins is briefly discussed.

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A COLORIMETRIC MICROMETHOD FOR THE DETERMINATION OF SODIUM WITH MANGANOUS URANYL ACETATE

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When a solution of manganous uranyl acetate in dilute acetic acid is added to a solution containing sodium ions, the sodium is quantitatively precipitated as sodium manganous uranyl acetate according to the following equation

$$Na^+ + Mn(CH_3COO)_2 + 3(UO_2)(CH_3COO)_2 + CH_3COOH + 6H_2O = NaMn(UO_2)_3(CH_3COO)_9 \cdot 6H_2O + H^+$$

This reaction is similar to that utilized by Barber and Kolthoff (1), who employed zinc uranyl acetate to precipitate sodium quantitatively as the triple salt sodium zinc uranyl acetate. The use of such reagents for precipitating sodium originated from an observation by Streng who in 1886, while working on the analysis of minerals, reported to Fresenius (2) that his uranyl acetate reagent for sodium was greatly improved after the addition of magnesia. Chang and Tseng (3) used manganous uranyl acetate in a qualitative test for sodium and determined the composition of the precipitate as given above.

In 1938 Woelfel (4) described a colorimetric method for the determination of sodium with this reagent. In his procedure, the precipitate was washed with a glacial acetic acid reagent, then treated with potassium periodate (5) to oxidize the manganese to permanganate, and the solution of permanganate thus obtained was compared with a suitable standard in a colorimeter. This method has several disadvantages and potential sources of error. These are due to the use of glacial acetic acid for washing the precipitate, to inconsistent values of the blank, and to the necessity

of preparing several standards. The method here described was designed to overcome these difficulties. In the new procedure the permanganate concentration is measured either by comparison with a standard solution of potassium permanganate in a colorimeter, or by direct measurement in the Evelyn photoelectric instrument (6). The blank is negligible. Large quantities of phosphate do not interfere.

Reagents—

1. 95 per cent alcohol.

2. Manganous uranyl acetate stock solution. To 160.0 gm. of $UO_2(CH_3COO)_2 \cdot 2H_2O$, 490.0 gm. of $Mn(CH_3COO)_2 \cdot 4H_2O$, and 138.0 cc. of 30 per cent acetic acid add 1292.0 cc. of water, to make approximately 2 liters of solution. *Pure manganous acetate should be in the form of pink lustrous crystals, free from grayish powder,* and should be stored in a brown bottle. It must be stressed that gray amorphous material labeled only "manganese acetate" cannot be used in the preparation of this solution. The salts dissolve readily at room temperature if the bottle is shaken occasionally. The less soluble crystals of uranyl acetate may be crushed from time to time with a stout, flattened glass rod. As the salts dissolve, sodium impurities produce a fine, crystalline precipitate of the triple salt throughout the solution. This precipitate settles out after a while, leaving a clear solution.

Woelfel's reagent, 25 per cent alcoholic manganous uranyl acetate solution, was found to decompose if it stood for a few weeks in an ordinary bottle exposed to sunlight. The odor of acetaldehyde of such a solution and the presence of a black precipitate make it seem probable that hexavalent uranium is reduced and the alcohol oxidized. An aqueous stock solution was therefore prepared and alcoholic dilutions were made from this solution as they were needed and kept in the dark.

3. 25 per cent alcoholic manganous uranyl acetate solution. For each sample to be analyzed, take 9 cc. of Reagent 2, add 3 cc. of 95 per cent alcohol, and mix. Cover and allow to stand, protected from direct light (in a cupboard), for 4 hours or longer. Filter the solution through a No. 42 Whatman or similarly retentive filter paper. The solution keeps for about 3 weeks if stored in the dark.

4. Sodium manganous uranyl acetate salt. In a beaker add

125 cc. of manganous uranyl acetate stock solution (Reagent 2) to 2 cc. of a 5 per cent sodium chloride solution. Stir with a glass rod and allow to stand for about ½ hour to allow precipitation of the sodium salt. Draw off the supernatant fluid and transfer the precipitate to a 50 cc. centrifuge tube. Centrifuge and remove the supernatant fluid by suction through a capillary tube. Wash the precipitate by resuspension and centrifugation three times with 95 per cent alcohol and twice with ether, removing the supernatant fluid each time by suction. Allow the ether to evaporate and store the triple salt in a brown vial.

5. Zinc uranyl acetate stock solution (Barber and Kolthoff's (1) reagent).

In the preparation of Reagents 2 and 5 it is satisfactory to mix the constituents and dissolve them together at room temperature, rather than to dissolve them separately on the steam bath and mix while still warm, as recommended for the zinc reagent by Barber and Kolthoff.

To 160.0 gm. of $UO_2(CH_3COO)_2 \cdot 2H_2O$, 440.0 gm. of $Zn(CH_3COO)_2 \cdot 4H_2O$, and 138.0 cc. of 30 per cent acetic acid add 1342.0 cc. of water, to make approximately 2 liters of solution. Dissolve the salts as directed for Reagent 2.

The zinc uranyl acetate solution prepared according to Barber and Kolthoff must stand for 24 hours before it is used. This is to allow the solution to cool to room temperature and to obtain equilibrium between the dissolved and solid phases of the triple salt. If the reagents are mixed and dissolved as described here, the solution is ready for use after 4 hours. Like Barber and Kolthoff, we have found sufficient sodium present in these reagents, as impurities, to form a saturated solution. This was found also in the reagents used in preparing the manganous uranyl acetate solution (Reagent 2). Numerous workers, using modifications of the Barber and Kolthoff method, report that solid sodium triple salt must be added in order to saturate the zinc uranyl acetate solution. If the reagents have insufficient sodium impurity for saturation, an easier way to saturate the solution is to add 0.1 N NaCl to the double acetates until a precipitate persists. Only a few drops of the sodium chloride solution are required.

6. 25 per cent alcoholic zinc uranyl acetate wash solution.

For each sample to be analyzed, take 12 cc. of zinc uranyl acetate solution (Reagent 5) plus 4 cc. of 95 per cent alcohol. Saturate this solution with *manganous* triple salt by adding 20 mg. of solid triple salt (Reagent 4), measured sufficiently accurately from the tip of a spatula, to each 100 cc. of this solution. Allow to stand 1 hour or longer with occasional shaking, and filter through Whatman No. 42 or similarly retentive filter paper. This solution keeps for at least 3 weeks in the dark. This reagent must not be contaminated with manganese Reagents 2 or 3.

7. Potassium periodate solution. Rinse 2.5 gm. of powdered KIO_4 into a 1000 cc. volumetric flask with about 400 cc. of water; add 100 cc. of 85 per cent phosphoric acid and shake the flask until the periodate is dissolved. Fill to the mark with water and mix. Keep the solution in a refrigerator. The solution turns slightly pink, probably on account of traces of manganese in the phosphoric acid, but this coloration does not affect the determination.

8. 20 per cent trichloroacetic acid. Dissolve 200 gm. of trichloroacetic acid, c.p., in water, dilute to 1 liter with water, mix, and filter, if necessary. Keep in a refrigerator.

9. Potassium permanganate stock solution. $KMnO_4$ crystals, dried to constant weight over sulfuric acid, must be standardized by titration with standard oxalate solution. The stock solution should be about 1.4 mM. Weigh accurately about 0.2200 gm. of the dried crystals; transfer these with water to a 1000 cc. volumetric flask, taking care that all crystals are dissolved. Add 200 cc. of periodate solution (Reagent 7), fill to the mark with water, and mix. If the solution is stored in a glass-stoppered brown bottle, protected from dust, it keeps for at least 6 months.

Procedure

For deproteinization with trichloroacetic acid, a dilution of 1:25 or more is used in order to minimize the error due to the mass of the protein precipitate.

Deliver 0.10 or 0.20 cc. of whole blood, serum, or urine into a 5 cc. volumetric flask containing 0.5 cc. of 20 per cent trichloroacetic acid. Fill to the mark with water and shake thoroughly. Foam may be dispelled by touching the surface with a fine wire dipped in caprylic alcohol. Filter. The trichloroacetic acid may

be omitted with protein-free materials. A blank, the blood omitted, may be carried through the procedure. If the reagents are pure, this blank should be negligible.

For duplicate analyses, transfer 1 cc. volumes of the clear, protein-free filtrate into conical 15 cc. (preferably graduated) centrifuge tubes. Add about 9 cc. of the 25 per cent alcoholic manganous uranyl acetate solution (Reagent 3) and mix well, with a fine glass rod, freshly cleaned with sulfuric-chromic acid mixture. The precipitate appears usually within a minute. Rinse the rod with about 1 cc. of the reagent. Cover the tubes with rubber caps and allow them to stand protected from direct light for at least 4 hours or, preferably, overnight. Centrifuge the tubes while covered with the rubber caps and remove the supernatant fluid by suction through a capillary tube.

To wash the precipitate and the inner wall of the tubes free of manganese, use about 4 cc. of washing solution (Reagent 6) delivered from a pipette, and suspend the precipitate in the washing fluid by stirring with a fine glass rod. Rinse the glass rod with about 1 cc. of washing fluid. Centrifuge the tubes again, remove the supernatant fluid, and repeat the washing twice as before. For the third washing, merely add 4 to 5 cc. of washing solution; resuspension of the precipitate in the washing solution is not necessary.

To evaporate the alcohol, place the tubes in water, raise the temperature of the water slowly to the boiling point, to prevent sputtering, and evaporate most of the remaining supernatant fluid in the tubes. If the heating is done under a hood, the alcohol will have completely evaporated after boiling for about 15 to 20 minutes.

Add about 10 cc. of KIO_4 solution (Reagent 7) to the tubes. Stir with a glass rod to dissolve the salt and place the tubes in boiling water for about 10 to 15 minutes. After cooling, transfer the solutions quantitatively to volumetric flasks. 25 or 50 cc. volumetric flasks, respectively, are used for 0.10 or 0.20 cc. samples of blood. Fill the flasks to the mark with water and mix.

The sodium concentrations are now determined by comparing the samples with a $KMnO_4$ standard in a comparison colorimeter, or by reading the per cent light transmission of the samples in the Evelyn photoelectric colorimeter.

For the comparison colorimeter, the $KMnO_4$ standard is prepared by diluting 4 cc. of the $KMnO_4$ solution (Reagent 9) to 50 cc. with water. If 0.10 cc. of sample was used, only 2 cc. are thus diluted. The standard is set at 20 mm.

Calculation—

$$\frac{20 \times \text{mM } KMnO_4 \text{ in standard} \times 1000}{\text{Reading} \times \text{cc. of sample precipitated}} = \text{mM sodium per liter}$$

For reading in the Evelyn photoelectric colorimeter, Filter 520 is used.

Calculation—Density[1] × factor = mM of Na per liter or mg. of Na per 100 cc.

This is derived from

$$\frac{\text{Density} \times 1000}{39.27 \times (100/(\text{volume oxidized solution})) \times \text{cc. sample analyzed}} = \text{mM Na per liter}$$

The following precautions, recommended by Evelyn, are especially necessary in this method. The hairline of the galvanometer must be at rest at the 0 position of the scale after the galvanometer lamp has been turned on. After the instrument has "warmed up" for about 1 hour, the position no longer shifts. Variations in the transmission readings of the Evelyn tubes for different positions in the socket must be excluded by calibration. A mark is scratched on the tube and readings are always taken

[1] Density, Evelyn's L value, is obtained from G, the corrected galvanometer reading, either directly from Evelyn's table or by the equation $L = 2 - \log G$. The value 39.27 (density of 1 mM $KMnO_4$ in 100 cc.) should be redetermined for each laboratory. To express the concentration of sodium in terms of mM per liter or as mg. per 100 cc., the density value may be multiplied with the appropriate factor from the following tabulation.

Blood serum or urine for trichloroacetic acid pptn.	After addition to trichloroacetic acid made up to	Trichloroacetic acid filtrate taken for analysis	Dilution after oxidation with KIO_4	Equivalent of original material	Factor for multiplying density of solution	
					For mM Na per liter	For mg. Na per 100 cc.
cc.	*cc.*	*cc.*	*cc.*	*cc.*		
0.10	5	1	25	0.02	318.3	732.1
0.20	5	1	50	0.04		
1.00	25	1	50	0.04		

with the calibration mark placed directly above the white line on the tube carrier.

EXPERIMENTAL

0.2300 gm. of potassium permanganate crystals, dried to constant weight over sulfuric acid, was dissolved in water and made to 1 liter. By standardization against oxalic acid, the solution was found to be 1.426 mM.

Permanganate has a minimum light transmission at wave-length close to 520 mμ. Therefore, Filter 520 supplied by the manufacturers of the Evelyn instrument has been used.

From the 1.426 mM potassium permanganate solution various dilutions were prepared and the per cent transmission readings made in the Evelyn photoelectric colorimeter. The densities plotted against the concentrations formed a straight line, going through the origin. The mean of eighteen values of the density of 1 mM permanganate per 100 cc. was 39.27. This factor is apparently not influenced by temperature changes between 15–27°. The presence of ions other than permanganate did not interfere. This is in agreement with the recent results of Mehlig (7).

Since the manganous triple salt contains 1 mole of Na per mole of Mn, the factor 39.27 has been used to calculate the sodium concentrations of the samples.

After these experiments with pure potassium permanganate solution, 0.1 to 0.5 mg. samples of sodium, from a standard sodium chloride solution, were precipitated, washed, and oxidized according to the method of Woelfel (4). The density values of these solutions were determined and their sodium equivalencies calculated. Losses of about 0.03 mg. of sodium had occurred. These losses were approximately constant, regardless of the size of the sample. The per cent error in the recovery of sodium, therefore, increased with decreasing amounts of sodium in the sample. This finding indicated that the loss of precipitate occurred during washing with glacial acetic acid reagent. Other washing solutions were then tried.

95 per cent and absolute alcohol, saturated with sodium manganous uranyl acetate, were found to be unsatisfactory because these reagents decomposed after standing for a few weeks, with the formation of higher oxides of manganese.

With a washing solution of equal parts of 95 per cent alcohol

and ether, in which the triple salt is practically insoluble, consistent results were obtained. The recovery of sodium from solutions of sodium chloride was, however, about 2 per cent too high (see Table I).

When any of these washing solutions was added to the manganous uranyl acetate solution remaining in the centrifuge tube, additional triple salt crystallized out, thus giving rise to a "blank." When 95 per cent alcohol and ether washing solution was used, the blank amounted to about 8 to 12 micrograms of sodium. If the tubes drained uniformly, the blank should be constant. But even tubes which had been thoroughly cleaned with hot sulfuric-

TABLE I

Per Cent Recovery of Sodium (0.03 to 0.27 Mg. Samples) from Solutions of NaCl and of NaCl Plus KH_2PO_4 (Molar Ratio Na:P = 11.0), after Washing Precipitates with Equal Parts of Alcohol and Ether or with 25 Per Cent Alcoholic Zinc Uranyl Acetate Solution (Reagent 6)

Material	Washing solution	No. of observations	Mean recovery of Na	S.D.*
			per cent	
NaCl	Equal parts 95% alcohol and ether	45	102.0	1.75
" + KH_2PO_4		20	101.3	1.60
"	25% alcoholic zinc uranyl acetate	18	100.7	1.8
" + KH_2PO_4		18	100.2	2.3

$$*\text{S.D.} = \sqrt{\frac{\text{sum of squares of differences of each observation from mean}}{\text{total No. of observations}}}.$$

chromic acid mixture frequently were found to retain drops of manganous uranyl acetate solution, especially near the mouth. The error due to this was minimized by wiping the tubes, around the mouth and inside to a distance of 2 to 3 cm., with cotton wound around a wooden stick and moistened with the alcohol and ether solution.

Because the blank with alcohol and ether as washing solution was too large and irregular, other attempts were made to find a more satisfactory washing solution. Finally, a 25 per cent alcoholic solution of Kolthoff's zinc uranyl acetate reagent, saturated with sodium *manganous* uranyl acetate, was tried and found satisfactory (Reagent 6). This solution has the same alcoholic

content as the precipitating reagent and, therefore, should not precipitate triple manganous salt from the supernatant solution remaining in the tube. Furthermore, its solubility for the manganous triple salt is so small that after the precipitate is washed the liquid remaining in the tube does not contain significant amounts of manganese. Therefore, draining and wiping are unnecessary. The size of the blank is negligible. As shown in Table I, this solution (Reagent 6) led to satisfactory recovery of sodium from solutions of sodium chloride.

Determination of Sodium in Presence of Phosphate—If manganous uranyl acetate solution (Reagent 6) is added to a solution of a sodium salt containing phosphate, uranyl phosphate is precipitated together with the triple salt. Although the precipitate of uranyl phosphate should not interfere with the determination of sodium in this method, Woelfel found that unless phosphate was removed, previous to the determination of sodium, too high results were obtained by his method. Data in Table I show that, with a molar ratio of Na:P = 11.0, P does not interfere with the determination by the method here described. In another set of experiments, 1 cc. samples of a standard sodium chloride solution were diluted with increasing amounts of a solution of phosphoric acid and made to 25 cc. volumes. The molar ratios of P:Na in these preparations ranged from 0 to 0.84. Sodium was determined in 1 cc. aliquots. The values for sodium found in this set of experiments varied by not more than ± 1.5 per cent from the theoretical, after correction for the blank due to the sodium impurity found in the phosphoric acid.

The acidity of the phosphoric acid did not affect the results, owing to the buffer action of the manganous uranyl acetate solution. In another experiment, 0.12 mg. of sodium, in the presence of 4 milliequivalents of H_2SO_4, was recovered without loss.

It may, therefore, be concluded that phosphate of blood, tissues, and urine need not be removed prior to the determination of sodium by this method.

In Table II values for sodium in urine, whole blood, and serum, determined by this method, are compared with values found by the gravimetric method of Butler and Tuthill (8). In the latter method phosphate was removed from urine and whole blood by powdered calcium hydroxide.

Influence of Potassium on Determination of Sodium—The influence of potassium on the determination of sodium was tested by analysis of a standard sodium chloride solution and of a urine low in potassium, to both of which various quantities of potassium

TABLE II

Comparison of Sodium Values in Urine, Whole Blood, and Serum

The results are expressed in milliequivalents of sodium per liter.

Material	Gravimetrically, Butler and Tuthill (8)	Manganous uranyl acetate method	
		Colorimeter	Photoelectric colorimeter
Urine	88.0	88.0	87.7
	168.2	170.5	170.1
	58.4	58.4	58.0
	76.4	76.0	75.7
	68.8	68.8	68.1
	32.5	32.8	32.8
	40.3	40.3	40.2
	16.2	16.3	16.3
Whole blood, normal	89.2		89.0
	83.4		83.3
	85.5		84.5
	97.0	98.0	97.0
	94.1	93.7	93.0
Serum, normal	142.2		141.3
	140.9		139.4
	139.0		138.0
	142.1		140.1
		144.4	143.6
		141.6	142.7
		147.6	146.9
Serum, Addison's disease	113.0	112.8	112.9
" " "		116.5	116.1
" " "	112.0	113.6	112.4
" " "	134.0	132.7	132.2

chloride solution were added. Sodium in the presence of potassium chloride was then determined by the colorimetric manganous uranyl acetate method and by the gravimetric zinc uranyl acetate method. For both methods it was found that, when the molar ratio of K:Na was 1.5 or less, there was no interference by potas-

sium chloride. With both methods, when 3 to 4 times more K than Na were present, the results were 3 to 4 per cent too high. With higher K:Na ratios, the errors increased sharply.

Manery and Hastings (9) who have recently reported high results with the gravimetric zinc uranyl acetate method, applied a correction of 3 to 5 per cent in the analysis of tissue. If, instead of applying a correction, one wishes to reduce the potassium concentration to a level where it does not interfere, potassium may be removed by precipitation as the perchlorate. In order to test the removal of potassium by this method, determinations of sodium were made on a sample of serum to which varying amounts of potassium chloride solution were added. The molar ratios of K:Na in the samples ranged from 2.0 to 7.0. The samples were deproteinized with trichloroacetic acid according to the procedure here described and aliquots of the protein-free filtrates containing at least 0.2 mM of potassium were evaporated to dryness on the steam bath. The residues were dissolved in 2 cc. portions of water and 1 cc. of a saturated solution of ammonium perchlorate was added to each. The $KClO_4$ crystals were removed by centrifugation and 2 cc. portions of the supernatant fluids were diluted with water to such a volume that 1 cc. contained approximately 0.1 mg. of sodium. The Na concentration was then determined as described above. The excess ammonium perchlorate was found not to interfere with the determination. The values for sodium found in these preparations were in close agreement with values for sodium found in the samples of serum without added potassium chloride.

Size of Sample and Concentration of Permanganate Solution—The data in Table I show the results of analyses of samples of sodium chloride ranging from 0.03 to 0.27 mg. of sodium. In order to have the readings of the galvanometer of the Evelyn instrument fall between 10 and 90 per cent, the volumes of the permanganate solutions should be 25 or 50 cc. for from 0.03 to 0.12 mg. of sodium (about 0.01 to 0.04 cc. of serum). For from 0.06 to 0.09 cc. of serum the volume should be 100 cc.

Usually 0.04 cc. of protein-free blood or serum (1 cc. of a 1:25 dilution) was employed and made to 50 cc. for colorimetric measurement.

The packed precipitate of a sodium sample of convenient size occupies about 0.15 cc. or less. If too large a sample has been precipitated, as sometimes may happen with urine, the precipitate may be dissolved in a suitable volume of water before oxidation, and part of this solution may be taken for oxidation with potassium periodate. If the solution of the triple salt, oxidized and made to volume, should prove to be too concentrated, part of it may be diluted with water to a suitable volume and then compared in the colorimeter or read in the photoelectric colorimeter.

Quantities of 0.008 to 0.01 mg. of sodium have been determined photoelectrically by this method with an accuracy of ± 8 per cent. The samples were allowed to stand overnight for complete precipitation of the triple salt, 5 cc. of potassium periodate were used for oxidation, and the solution was made to 10 cc. for colorimetric measurement. Although no special precautions regarding temperature were found necessary in the determination of sodium by this method, care was taken to saturate the precipitating and washing reagents with the triple salt at approximately the same temperature as that at which the sodium precipitate was centrifuged.

The precipitating reagent might be improved by increasing the alcohol concentration to the maximum that would leave sufficient manganous uranyl acetate in the solution. A precipitating reagent containing 50 per cent alcohol by volume followed by a washing solution of the same alcoholic content has been found satisfactory.

SUMMARY

A micromethod for the determination of sodium is described. Sodium is precipitated by manganous uranyl acetate as the triple acetate, and the precipitate is washed with a solution of zinc uranyl acetate saturated with the manganous triple salt and having the same alcoholic concentration as the precipitating reagent. The precipitate is treated with potassium periodate and the solution of permanganate thus obtained is measured by comparison with a standard of potassium permanganate in a colorimeter or directly in the Evelyn photoelectric colorimeter. Phosphate in biological material does not interfere. Potassium causes high results if the molar ratio of K:Na in the sample exceeds 1.5.

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PURIFICATION OF THE CHROMATOPHOROTROPIC HORMONE OF THE CRUSTACEAN EYESTALK*

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The chromatophorotropic hormone of the crustacean eyestalk was discovered in 1928 by Perkins (1) and somewhat later by Koller (2). This hormone was characterized as a water-soluble, heat-stable substance, effective over a wide dilution range. Very little further information concerning the chemistry of this hormone was added during the next 10 years, most of the attention being directed to its physiological properties. Recently, Carlson (3) and Abramowitz (4) added further properties—its solubility in the lower alcohols, its insolubility in most common organic solvents, its relatively small molecular size, and its behavior towards acid and basic hydrolysis. The introduction of a method of biological assay for this hormone by Abramowitz (4) and Abramowitz and Abramowitz (5) afforded a more quantitative treatment which had been lacking, and the purification of the material was attempted on the basis of its known properties, by fractional precipitation from alcoholic solutions (4, 5). Only slight progress was made, however, owing to the obvious limitations of such a method in dealing with a substance of unknown chemical nature.

Since the eventual isolation of this physiologically powerful substance will depend partly on the recognition of its chemical nature, the problem was reinvestigated, and the results are presented in this paper. A study of the reactions of this hormone, which will be described briefly below, has indicated that the material is probably a nitrogenous base, and a valuable purification has been effected upon the basis of its basic properties.

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Methods

The tissue used for extraction was the eyestalks of the fiddler crab, *Uca pugilator*. The eyestalks were cut from hundreds of animals and kept at 3° until sufficient material was accumulated. The dry weight of an average eyestalk is 2 mg., and consequently 250 animals had to be operated on to obtain a gm. of tissue. Usually, 0.5 to 1.0 gm. of material was worked up at one time. The method of gaging the amount of hormone was that described by Abramowitz and Abramowitz (5).

EXPERIMENTAL

Standard Tissue Powder—Several eyestalks were dried, pulverized, and the powder extracted three times with distilled water and the extract assayed. The activity of these extracts in terms of the weight of the powders used varied from 6000 to 1000 *Uca* units per mg., depending on the freshness of the material, size of the animals from which the eyestalks were removed, etc. When larger amounts (0.5 to 1.0 gm.) of eyestalks were powdered, the activity averaged 2000 *Uca* units per mg. This value was taken as a comparison standard.

Extraction—1 gm. of eyestalks was ground thoroughly with 15 cc. of distilled water. The soluble portion was decanted, and the residue reextracted twice in the same fashion. The combined extracts (45 cc.) were heated to 100° for 5 minutes, and filtered. The residue was washed with 5 cc. of water, and water was added to the filtrate and washings to make 50 cc. of solution. 1 cc. of solution then contained the equivalent of ten eyestalks. About 30 per cent of the solid material and practically all of the activity were extracted. The aqueous extract (pH 6.0 to 6.5) was slightly turbid, orange in color, with a total activity of 2×10^6 *Uca* units, or about 6000 *Uca* units per mg. Attempts to improve the yield in terms of activity per mg. of solids by initial extraction with dilute alcohol (60 to 80 per cent) were unsuccessful, nor did more acidic extracts improve the yield significantly.

Fractionation with Barium Hydroxide or Lead Subacetate—Recrystallized barium hydroxide was added to the aqueous extract until the pH of the solution was 8.0 to 8.5. The copious precipitate which resulted was discarded after centrifuging. The supernatant solution (colored lemon-yellow) contained

practically all of the activity. Excess barium was removed as the sulfate, and the solution brought to neutrality.

The aqueous extract can also be fractionated with lead subacetate, which was added to the initial extract until no further precipitation occurred. More inert material is brought down than with barium hydroxide. After centrifugation to remove the lead salts, the supernatant solution (possessing a greenish yellow tinge) was treated with sulfuric acid to remove excess lead, and adjusted to neutrality with saturated sodium hydroxide.

Adsorption—Washed fullers' earth (0.5 gm.) was added to the neutralized solution with stirring. After several minutes, the solution was centrifuged and the adsorbate rejected. A slight loss in activity occurred at this stage but it was not appreciable. The supernatant solution was now colorless and water-clear, and still possessed practically all of the activity originally present.

Formation of Silver Salt—A slight excess of silver nitrate was added to the solution previously brought to pH 2.5 to 3.0 with nitric acid. The resulting precipitate of silver salts was discarded after centrifuging, and the supernatant solution brought to pH 8 to 9 with saturated baryta water, and centrifuged. The precipitate was dissolved in nitric acid at pH 3.0 with gentle warming. Solution is almost complete and, after rejection of the insoluble material, the solution was brought to pH 7.2 with baryta. The precipitate, which contained most of the activity, was decomposed with 10 per cent hydrochloric acid in slight excess, and the solution diluted to 10 cc. with water and centrifuged free of silver chloride. The latter was washed with small amounts of distilled water and the washings added to the main solution containing the activity. The combined solution was adjusted to pH 7.0 with saturated sodium hydroxide and centrifuged free of insoluble material. The supernatant solution contained about one-third of the activity originally present.

Formation of Phosphotungstate—The solution (20 cc.) was adjusted to pH 2.8 with sulfuric acid, and the sulfates removed. A few pinches of phosphotungstic acid crystals were added with stirring to the supernatant liquid, and the solution placed at 0° for a few hours. The phosphotungstates were removed by centrifuging and decomposed with small amounts of cold, saturated barium hydroxide. The soluble portion was decanted, and

the residue triturated two more times in the same fashion, and then discarded. The combined solutions (5 cc.) were then adjusted with sulfuric acid to pH 7.5, and the sulfate removed.

Extraction with Alcohol—The slightly alkaline solution of the hormone was added to 95 cc. of ethyl alcohol with stirring, and centrifuged. The insoluble material was discarded, the excess barium was removed from the supernatant solution as the carbonate, and the alcoholic solution evaporated to dryness. The residue is a hard, white crystalline mass containing both organic and inorganic material. Extraction of this residue with small amounts of hot alcohol and evaporation of the extracts yielded smaller residues of the brittle crystalline mass. The activity of the material from this and other batches worked up in similar fashion was from 200,000 to 400,000 *Uca* units per mg. This represents a 100- to 200-fold increase in purity, and retains about 10 to 20 per cent of the activity originally present.

Other Properties—The activity of the various fractions has been given in round figures, since the biological method of assaying the extract is not very accurate. It is, however, the only method at present of following the activity. Another difficulty was the minuteness of the purified material for dry weight determination. All of the final solution had to be dried in order to obtain sufficient material that could be weighed without great error. The greatest difficulty, however, lies in the paucity of material for extraction and in the minute amounts of hormone present in the eyestalk.[1] Consequently, it is difficult to denote the above reactions in unqualified terms as properties of the hormone itself and not artifacts due to the adsorption of the hormone to other larger constituents of the extract. However, the various steps have been performed singly on crude extracts, and in varying sequence, and it can be stated with a good deal of assurance that the activity has always been found as stated in but one respect. The

[1] Of interest is the rough calculation of Abramowitz and Abramowitz (5) that one eyestalk of *Uca* contains about 0.2 microgram of hormone. A much better starting material than the whole eyestalk would naturally be the sinus gland of the eyestalk, the minute gland which produces the hormone. The initial aqueous extract of the gland would be 100 or more times purer than that of the entire eyestalk, but it would be an almost herculean task to obtain even a gm. of sinus gland tissue.

silver fractionation has not been found to give constant results. The purine fraction is always mainly inactive. It is, however, sometimes necessary to saturate the solution with baryta to bring down most of the activity. Proline fractions are always active though slightly so. The histidine fraction was usually taken because of the slight precipitate in comparison with that of the combined histidine and arginine silver fractions.

The purified solutions formed good precipitates with gold chloride in the presence of hydrochloric acid. With mercuric acetate in alcoholic medium, it forms a white granular precipitate. Amorphous precipitates are formed with flavianic and picrolonic acids. The activity of the solutions treated in these ways must go with the precipitate, since the supernatant material is mainly inactive. A good deal is lost, however, especially in the formation of the gold salt, because complete recovery has not been obtained after decomposition.

Physiological Properties—There is considerable difference of opinion concerning the number of chromatophorotropic hormones[2] produced by the eyestalk, varying estimates of from one to nine or more having been made. The consensus of workers appears to favor several hormones in operation, but the final answer to this problem can be given only by the separation or isolation of the various principles. The purified material was injected into *Palæmonetes* and *Crago* in a concentration of 1 eyestalk per cc. of solution. Both specimens responded promptly by the formation of the pale phase. Thus, no qualitative separation had occurred, but it must be emphasized that this result can have no bearing on the question, since the material, though in a purer state than has been hitherto obtained, is still far from being pure. The observation does indicate, however, that if several chromatophore hormones are present, they are probably closely related substances.

SUMMARY

The chromatophorotropic hormone, or hormones, in the eyestalks of *Uca pugilator* reacts in a characteristic fashion for amino bases. It has been purified 100- to 200-fold by precipitating inert

[2] See the review by Abramowitz (6).

material with barium hydroxide or lead subacetate from initial aqueous extracts. Further impurities are removed by adsorption. The activity is precipitated in the histidine silver fraction, and then converted into the phosphotungstate. After decomposition of the latter, the hormone is dissolved in alcohol in a slightly alkaline medium and dried.

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ANEMIA IN CHICKS CAUSED BY A VITAMIN DEFICIENCY*

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Hogan, Boucher, and Kempster (1) succeeded in formulating a simplified ration for the chick which for many months seemed to be entirely adequate. In later studies, however, the earlier success could not always be duplicated, and eventually it developed that the inconsistencies were due to variability of the commercial liver extracts employed. An attempt was made to eliminate the source of this difficulty, and while these studies were in progress it was observed that some of the chicks were severely anemic. It seemed clear that this was a deficiency disease, and that the antianemic vitamin is not identical with any other now recognized.

The purpose of this investigation was (1) to devise a technique by which the anemic condition is produced consistently and (2) to observe alterations in the red blood cells that might be associated with the anemic condition.

EXPERIMENTAL

Day-old single comb white Leghorn chicks were used exclusively, and all rations were of the simplified type. The cages and method of handling are described in an earlier publication (2).

The liver extract in use when the first cases of anemia developed was the portion of the water extract of liver which is soluble in 70 per cent alcohol, and it is used in the treatment of pernicious anemia. When the anemia was first observed, only a small quantity of the preparation then in use was available, and all attempts

* Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series, No. 623.

to repeat the observation with subsequent shipments failed completely. It was finally decided to prepare the extracts in the laboratory, with pork liver as the raw material, and by the procedure presumably followed in the manufacture of the commercial product. The first trials with these were encouraging. The mortality was not excessive and severe anemia developed in 80 per cent of the chicks. The second preparation of this extract, however, did not induce anemia and it was decided to modify the procedure in the hope that the inconsistencies might be eliminated. It seemed probable that the anemia-preventing factor is relatively

TABLE I

Composition of Rations

	Ration 3738	Ration 3835*
	per cent	*per cent*
Casein	35	35
Corn-starch	35	42
Salts	4	4
Cellophane (Cellu flour)	3	3
Lard	8	7
Cod liver oil	2	2
Wheat germ oil	4	4
Acid-hydrolyzed yeast	4	
Ether extract of egg yolk	2	
Tikitiki	1	1
Liver extract (No. 3703)	2	2

* In addition each chick received daily 40 γ of crystalline thiamine and 20 γ of crystalline riboflavin.

insoluble in alcohol, so in subsequent trials the fraction of the liver extract was used which is soluble in 80 per cent alcohol. The results were again inconsistent, so it was decided to use still higher concentrations. Fresh liver contains approximately 70 per cent of water and it is impractical to add enough commercial 95 per cent alcohol to make the final concentration as high as was desired. In order to avoid this difficulty the liver was dried before beginning the extraction. The fresh material was ground, spread in thin layers, and dried at a temperature below 70°. The dry material was reground, extracted with 95 per cent alcohol at 70°, and the extract was filtered while hot. Some of the lipids

separate out on cooling and these were filtered off and discarded. The filtrate was concentrated *in vacuo* to a dry matter content of approximately 50 per cent. This preparation, No. 3703, is superior to any other that has been investigated thus far. The rations we have used most successfully are described in Table I.

Ration 3738 is a modification of the synthetic diet used by Hogan and Boucher, and it seemed probable that it is more complex than is necessary. Ration 3835[1] is simpler, but has not been used extensively enough to show decisively whether it is as satisfactory as Ration 3738. At the time the data for this report were assembled a total of 111 chicks, from several different hatches, had been supplied with these two diets. The mortality was 9 per cent and the incidence of anemia among the survivors was 100 per cent. In the interval between assembling the data and completing the manuscript a few additional chicks have been observed. Of sixteen chicks that received Ration 3738, there were four that did not survive long enough to become anemic. Of fifteen that received Ration 3835, only five survived long enough to become anemic. According to our tentative hypothesis Preparation 3703 is also deficient in at least one other unrecognized vitamin required by the chick, and if this deficiency is sufficiently acute the chicks die before observations can be made. The next problem is to develop methods of preparing extracts that are relatively free of the antianemic agent, but can be relied on to contain reasonable amounts of the other essential vitamins.

Observations on Blood of Anemic Chicks

Red Blood Cell Count—Doyle, Mathews, and Roberts (3) state that chicks, when confined, have red cell counts of from 2.0 to 2.3 millions per c.mm. Kelly and Dearstyne (4) report that the number of red cells ranges from 2.02 to 3.49 millions per c.mm., but in the greater number of cases the count falls between 2.27 to 3.01 millions per c.mm. Cook (5) included counts made by three other investigators, as well as by himself, and concluded that an average red blood cell count of 2.5 millions per c.mm. may be accepted as normal. This value is in satisfactory agreement with the average count obtained in this investigation. The averages of all observations which relate the erythrocytes to blood

[1] The data on this ration were supplied by Dr. L. R. Richardson.

volume are shown in Table II. Chicks with a red cell count below 2 millions per c.mm., unless it increased the following week, were considered anemic. In most cases a red cell count of 2 millions would be followed by progressively lower values each week until the chick succumbed.

Per Cent of Hemoglobin—In the preliminary studies the hemoglobin estimations were made with a Dare hemoglobinometer, but this method was soon supplanted by the procedure of Schultze and Elvehjem (6). Holmes, Pigott, and Campbell (7) reported that at the age of 6 weeks the blood of Rhode Island Red cockerels contains 9.7 gm. of hemoglobin per 100 cc., and the blood of pullets contains 9.6 gm. Harmon (8) did not mention the breed used, but reported that at 4 weeks of age the mean per cent of hemoglobin in the blood of cockerels is 8.62, of pullets 8.80. At 8 weeks the percentages had risen to 9.78 for males, and 9.54 for females. Both reports indicate, as do our own studies, that in the early weeks of a chick's life the percentage of hemoglobin is affected little or not at all by sex.

Elvehjem and Hart (9) also used white Leghorns, but did not mention the sex or weight. The chicks were first made anemic by providing them with a ration deficient in iron, and then in a study of the anemia-preventing substance they were supplied with various iron compounds. The analyses indicate that a hemoglobin percentage of approximately 8 is a normal value, though in many groups the values were considerably lower. Hart, Elvehjem, Kemmerer, and Halpin (10) reported on the average hemoglobin content at various ages of the blood of nine groups of chicks which received practical chick rations. The highest average reported at 57 days was 7.14 per cent; the lowest was 6.22. The mean of our observations on chicks which received a normal ration was 7.36. A chick is regarded as definitely anemic if the percentage of hemoglobin falls below 5.

Volume of Red Blood Cells—As will be shown later, the red cells of anemic chicks are larger than normal but since the decrease in number proceeds far more rapidly than the increase in size, the relative volumes of the packed cells are also a reliable index of the degree of anemia. As is shown in Table II, in severely anemic chicks this value is about 30 per cent of that of normal chicks. The Wintrobe (11) hematocrit tubes were used in making these estimates.

Average Red Blood Cell Hemoglobin—Since the majority of the red cells of the anemic chick is above the normal size, the actual amount of hemoglobin per cell is also larger than normal. This would be true even though the concentration of hemoglobin per cell were the same in each case. As is pointed out below though, the concentration of hemoglobin in the cells of anemic chicks is increased. This, with other observations, is shown in Table III.

Mean Red Blood Cell Hemoglobin Concentration—Estimates of the amount of hemoglobin per cell show that it is markedly increased in anemic chicks. An analogous situation was found by Dallwig, Kolls, and Loevenhart (12) while studying the effects of low oxygen tension on dogs, rabbits, and rats. In most of their experiments there was an increase, 30 per cent in some cases, in the amount of hemoglobin per red corpuscle in response to a decrease in partial pressure of oxygen, both at atmospheric and at reduced barometric pressure.

TABLE II

Weights of Chicks, and Relation of Erythrocytes to Blood Volume

Type of ration	Age	Weight	Red blood cells per c.mm.	Hb per 100 cc.	Red blood cell volume
	wks.	*gm.*	*millions*	*gm.*	*per cent*
Normal	6	381 ± 35	2.7 ± 0.29	7.4 ± 0.32	31.7 ± 1.39
Anemia-	4	93 ± 13	1.8 ± 0.41	6.3 ± 0.88	23.7 ± 4.63
producing	6	129 ± 23	0.7 ± 0.37	3.4 ± 1.46	10.8 ± 5.61
	8	167 ± 30	0.5 ± 0.05	2.9 ± 0.34	8.0 ± 1.28

Mean Red Blood Cell Width and Mean Red Cell Length—These measurements were taken on cells, chosen at random, from a blood smear stained with Wright's stain. Very few measurements of red blood cells of the chick are available. Kaupp (13) states that the average diameter (width) of the red cell is 7 to 8 μ, and the average length is 12 to 13 μ, though these figures vary with different breeds. Kelly and Dearstyne (4) report that they range from 7 to 9 μ in width and 11 to 15 in length. From the measurements shown in Table III it is evident that the red cells of anemic chicks are larger than those of normals.

Mean Red Blood Cell Volume—Since the red cells of anemic chicks are both longer and wider than those of normal chicks, one would expect that the volumes would also be greater than is

normal. At 4 weeks of age the red cells of chicks on anemia-producing rations were 25 per cent, and at 6 weeks they were 33 per cent above the normal size.

Fragility of Red Corpuscles—Destruction of red corpuscles goes on continually within the body and in certain pathological conditions this destruction is greatly accelerated, leading usually to

TABLE III

Individual Red Blood Cells

Type of ration	Age	Hb per cell Weight	Hb per cell	Length	Width	Volume
	wks.	γ	*per cent*	μ	μ	*cu. microns*
Normal	6	28 ± 0.71	24 ± 0.49	12.1 ± 0.58	6.8 ± 0.27	119 ± 3.56
Anemia-	4	36 ± 5.96	26 ± 2.66	13.6 ± 0.59	7.3 ± 0.69	147 ± 16.57
produc-	6	47 ± 9.84	31 ± 3.96	14.2 ± 0.66	7.2 ± 0.39	160 ± 33.04
ing	8	53 ± 8.91	36 ± 3.28	15.0*	6.8*	149 ± 26.86

* Only one chick.

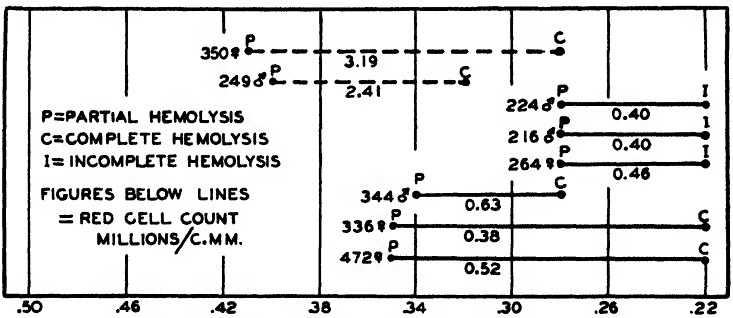

FIG. 1. The erythrocytes of normal chicks, indicated by broken lines, are much less resistant to hemolysis than those of anemic chicks, indicated by solid lines.

anemia. It seemed desirable therefore to ascertain whether or not the red blood cells of the anemic chick are excessively fragile, as is typically the case in human hemolytic jaundice, or less fragile, as is the case in most other anemias. Fragility determinations were made by the method of Sanford, as described by Todd and Sanford (14). As shown in Fig. 1, the red cells of the anemic

chicks are more resistant to hemolysis than those of normal chicks.

Blood Coagulation Time—A tendency towards delayed blood clotting time in chicks on certain synthetic rations was observed by Dam (15) and by McFarlane and coworkers (16), and later this tendency was attributed by Dam (17) to a deficiency of vitamin K. Since according to Almquist and Stokstad (18) the primary feature of a lack of this vitamin is the prolonged blood clotting time, it seemed desirable that some such determinations be made on the blood of the anemic chicks in this investigation. The blood coagulation time was obtained by placing several drops of blood (obtained with a platinum loop from a wing vein puncture) on a

TABLE IV

Coagulation Time of Blood

Per cent Hb	Anemia-producing ration			Control ration		
	No. of chicks	No. of readings	Coagulation time	No. of chicks	No. of readings	Coagulation time
			min.			*min.*
2.0-2.9	6	9	2.6			
3.0-3.9	4	7	2.1			
4.0-4.9	9	14	2.5			
5.0-5.9	2	4	2.0			
6.0-6.9	6	8	3.2	3	3	1.4
7.0-7.9	3	5	2.2	10	18	3.0

porcelain plate and drawing a needle through a drop at 30 second intervals. Zero time was recorded when the blood was placed on the plate and coagulation was considered as complete when the entire drop adhered together in a firm mass.

Table IV gives some of the values obtained and demonstrates that the blood coagulation time of the anemic chicks is not prolonged. All values obtained fall well within those for chicks on a stock ration.

Anemia Not Due to Fasting—It is well known that the number of erythrocytes may be reduced as a result of fasting, but it did not seem probable that the anemia observed in this investigation was due to inanition, for in many cases there was a gain in weight with a simultaneous decrease in the number of erythrocytes.

The relation of partial inanition to the anemia was studied in a more direct manner, on chicks that had been made anemic by supplying them with Ration 3738. As a control one-half of the group was continued on Ration 3738 *ad libitum.* The others were given just enough of a ration composed of natural foodstuffs,

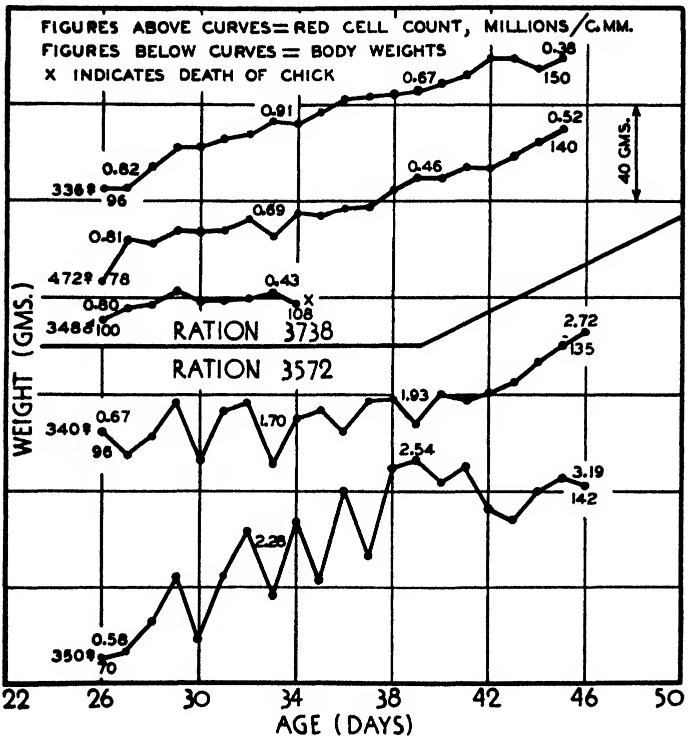

FIG. 2. The red blood cell count of chicks on Ration 3738 continued to decline. The rate of growth of chicks on Ration 3572 was retarded by limiting their food supply, but they recovered from anemia.

Ration 3572,[2] to keep them at the same weight as those on the anemia-producing diet. The erythrocyte counts in the control group continued to decline, while those in the group that received

[2] Ration 3572 contained whole wheat 57.6, casein 12.3, whole milk powder 8.2, alfalfa meal 2.5, butter fat 4.2, cod liver oil 1, yeast 12, NaCl 0.9, and $CaCO_3$ 1.3.

Ration 3572 returned to normal. This demonstrates again that the anemia is not a direct consequence of an insufficient food supply. The data are summarized in Fig. 2.

Nature of Antianemic Factor—It is impossible as yet to make any estimate of the number of unrecognized vitamins that are required by the chick but of those now recognized Jukes (19) lists nine, including vitamins A, B_1, D, E, K, anti-gizzard erosion factor, antiencephalomalacia factor, and G (flavin and the filtrate factor). More recently Pappenheimer *et al.* (20) have reported that the antiencephalomalacia factor is vitamin E, so the number is reduced to eight. According to recent reports vitamin K (21), the filtrate factor (22, 23), and the anti-gizzard erosion factor (24, 25) have also been identified.

Presumably all the above factors have been included at some time or other in the rations used in this investigation. Vitamins A and D were supplied by cod liver oil; vitamin B_1 by tikitiki and liver extracts, and at times by crystalline thiamine; vitamin E was supplied in wheat germ oil; vitamin K by liver extracts from which the fatty portion had not been separated by ether, by ether extract of egg yolk, and by ether extract of alfalfa. Furthermore, hemorrhages were never observed and clotting time determinations, made at intervals throughout the investigation, failed to reveal any abnormality.

Riboflavin was supplied in the liver extracts, although the amount supplied by these extracts may have been suboptimal. The addition of crystalline riboflavin increased rather than decreased the incidence of anemia, owing to the fact that these chicks grew at a more rapid rate.

It has been assumed that the anti-gizzard erosion factor is not related to the antianemic vitamin, because gizzard erosions have been observed in many chicks that were not anemic. Furthermore the erosions were occasionally observed in chicks that had received rations which prevent anemia, or permit prompt recovery after it has developed.

There is also no reason now to suppose that the filtrate factor, or pantothenic acid, is the antianemic vitamin. Dermatitis was never observed in any of the chicks, whether they became anemic or not. Furthermore, according to Elvehjem and Koehn (26) this substance is somewhat soluble in 95 per cent alcohol,

and it is not adsorbed on fullers' earth. Our unpublished observations show that the antianemic agent is adsorbed on fullers' earth at a pH of 1. Jukes (27) has reported that the filtrate factor is soluble in 99.5 per cent ethyl alcohol, and his method of preparing crude preparations shows that it is not adsorbed on fullers' earth. As additional evidence it may be stated that tikitiki, prepared by the method of Wells (28), contains little or none of the antianemic agent, though the publication of Jukes (27) indicates that it should be an excellent source of the filtrate factor.

There is no evidence as yet that the chick requires nicotinic acid, but it seems certain that this vitamin does not prevent this type of chick anemia. Four anemic chicks did not improve when supplied with 0.65 mg. daily of this compound.

It is becoming increasingly difficult to select a suitable symbol for new vitamins, and a descriptive name is cumbersome. For convenience the factor that prevents the type of anemia described in this paper is designated as the B_c vitamin. It belongs to the old vitamin B complex and the small *c* is added because this substance is essential for the chick.

SUMMARY

When chicks receive a ration which contains the well recognized vitamins, and in addition a 95 per cent alcohol extract of liver, they grow slowly and become anemic.

The red blood cell count, the per cent of hemoglobin in the blood, and the red cell volume are all decreased.

The amount of hemoglobin per cell is increased.

The red blood cells of anemic chicks are longer and wider than is normal and the volume of individual red cells is increased.

The red blood cells of anemic chicks are less fragile than is normal.

The blood of anemic chicks clots in normal time.

The anemia is not due to fasting.

The antianemic agent could not be identified with any vitamin previously described.

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THE EFFECT OF PICRIC AND FLAVIANIC ACIDS ON THE POTENCY OF THE FOLLICLE-STIMULATING ANTERIOR PITUITARY HORMONE

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In a recent publication Fevold reported that the follicle-stimulating hormone is precipitated and reversibly inactivated by the addition of picrolonic, picric, or flavianic acid. After removal of these acids the follicle-stimulating hormone was precipitated with tannic acid and was injected as an insoluble tannate (1). Fevold also found that the luteinizing hormone[1] is not inactivated by these protein precipitants. We have previously employed picric and flavianic acids in our attempts to achieve a chemical separation and purification of these two gonadotropic principles. We found, however, that the physiological activity of the picrates and flavianates was not impaired. In this communication we wish to present our data pertaining to the effect of picric and flavianic acids on the potency of gonadotropic fractions obtained from sheep pituitary.

EXPERIMENTAL

The animals used for the physiological standardization of the various preparations were 21 to 23 day-old normal female rats, three to six rats being used in each group. They received one subcutaneous injection daily for 3 days. Autopsies were performed 72 hours after onset of treatment and the degree of ovarian and uterine development, as well as ovarian weights, was recorded.

The gonadotropic fractions used in these studies were a 40 per cent alcohol extract from whole sheep pituitaries containing

[1] It has been shown recently (2) that the luteinizing hormone fraction is identical with the interstitial cell-stimulating hormone.

both follicle-stimulating and luteinizing hormones, two follicle-stimulating hormone preparations of different potency, and a luteinizing hormone fraction. Their preparation and standardization in terms of rat units were carried out according to the methods previously published (2, 3). The tannates of the various fractions were obtained by adding a solution of tannic acid to the aqueous hormone solutions (pH 6) and the resulting suspension was injected as such.[2] The picrates and flavianates were prepared by adding half the amount of acid, dissolved in water, to the aqueous hormone solutions (pH 6).[3] The precipitates were allowed to settle overnight, removed by centrifugation, washed, and dried with alcohol and ether. These fractions have been designated as "water-insoluble" preparations. The supernatants were poured into absolute alcohol, the final alcohol concentration being 85 per cent. The resulting precipitates were centrifuged off, dried with alcohol and ether, and have been designated as "water-soluble" fractions. For the biological tests the various preparations were administered as follows: (*a*) dissolved in water at pH 7.2 and given alone, (*b*) combined *in vitro* with 10 rat units of chorionic gonadotropin (follutein, 0.05 mg., total dose per rat), and (*c*) combined *in vitro* with twice the amount of tannic acid except for the experiment recorded in Table III in which 1 mg. of tannic acid was used. All injections were given subcutaneously.

Results

The augmenting effect of tannic acid on different gonadotropic preparations is illustrated in Table I. This augmentation is generally considered to be due to a prolongation in the rate of absorption (4). Yet the effect of tannic acid on the response of the two gonadotropic hormones and their combination is markedly different.

Table II shows the gonadotropic effect of an unfractionated sheep pituitary extract and its water-insoluble and water-soluble picrates and flavianates. It allows a comparison of the augmentation ef-

[2] The amount of tannic acid used was usually twice that of the gonadotropic preparation.

[3] No further precipitation occurred when the amount of the reagent was increased.

fect of tannic acid with the specific synergism of chorionic gonadotropin. We have found that precipitation with either picric or flavianic acid is incomplete and that the material recovered from

TABLE I

Effect of Tannic Acid on Gonadotropic Potency of Sheep Pituitary Preparations

Six rats were used in each group.

Preparation, total dose	Alone			Plus tannic acid		
	Ovarian weight	Size of follicles	No. of rats showing corpora lutea	Ovarian weight	Size of follicles	No. of rats showing corpora lutea
	mg.			*mg.*		
40% alcohol extract, 2 mg.	39	Medium	2	106	Large	6
Same, 1 mg.	28	"	0	55	"	6
Follicle-stimulating hormone, 8 rat units (0.8 mg.)	30	"	0	43	Medium and large	3
Same, 4 rat units (0.4 mg.)	29	"	0	35	Large (4 rats)	1
Interstitial cell-stimulating hormone, 20 rat units (1 mg.)	18	Small	0	29	Medium large (5 rats)	3
Same, 10 rat units (0.5 mg.)	16	"	0	19*	Few medium large (6 rats)	0
Follicle-stimulating hormone, 4 rat units (0.2 mg.) + interstitial cell-stimulating hormone, 20 rat units (1 mg.)	31	Medium	2	53	Large (5 rats)	2
Follicle-stimulating hormone, 4 rat units (0.2 mg.) + interstitial cell-stimulating hormone, 10 rat units (0.5 mg.)	27	Large	2	52	" (5 ")	3

* Estrous uterus in four rats.

the supernatants is of high follicle-stimulating potency. No attempt has been made to evaluate, on a quantitative basis, the distribution of the active material between the water-insoluble

and water-soluble substances. It may be noted that the picrate fractions seem to be more potent per mg. than the starting material, which may be due to a different rate of absorption.

The gonadotropic effects of two follicle-stimulating preparations and their various picrate and flavianate fractions are illustrated in Tables III and IV. As indicated in the combination tests with chorionic gonadotropin, the follicle-stimulating

TABLE II

*Effect of Picric and Flavianic Acids on Gonadotropic Potency of Unfractionated Sheep Pituitary Extract**

Preparation, total dose	Alone		Plus 10 rat units follutein†	Plus 2 mg. tannic acid
	Ovaries	Size of follicles	Ovaries (with large follicles and corpora lutea)	
	mg.		*mg.*	*mg.*
Sheep extract, 1 mg.	27	Medium only	58	49
Picrate, water-insoluble, 1 mg.	47	Large‡	83	69
" water-soluble, 1 mg.	31	Medium only	116	64
" (insoluble) 0.5 mg. + picrate (soluble) 0.5 mg.	28	Large‡	95	66
Flavianate, water-insoluble, 1 mg.	26	Medium	81	46
" water-soluble, 1 mg.	21	Large	61	43
" (insoluble) 0.5 mg. + flavianate (soluble) 0.5 mg.	26	Medium and large	93	55

* 40 per cent alcohol extract of acetone-dried whole sheep pituitary.

† 10 rat units of follutein gave 30 mg. ovaries containing a few corpora lutea.

‡ Corpora lutea present.

hormone shows no signs of being inactivated by the addition of picric or flavianic acid. Again, the supernatants, precipitated in alcohol and tested as the "water-soluble" fractions, prove to be highly active.

It may be mentioned in this connection that we occasionally observed that the follicle-stimulating potency of sheep pituitary preparations was decreased after the addition of flavianic acid and we, therefore, abandoned the use of this reagent. We have

no explanation for this observation except that it may have been caused by too high a degree of acidity. But we wish to emphasize that the occasional partial destruction of the follicle-stimulating hormone by flavianic acid is irreversible. With picric acid

TABLE III

Effect of Picric and Flavianic Acids on Gonadotropic Potency of Hypophyseal Follicle-Stimulating Hormone

Preparation, total dose	Alone		Plus 10 rat units follutein*	Plus 1 mg. tannic acid	
	Ovaries	Size of follicles	Ovaries (with large follicles and corpora lutea)	Ovaries	Size of follicles
	mg.		*mg.*	*mg.*	
Follicle-stimulating hormone, 4 rat units (0.2 mg.)	21	Medium	81	38	Large
Picrate, water-insoluble, 0.4 mg.	19	"	109	21	Medium large
Picrate, water-soluble, 0.4 mg.	32	" large	91		
Flavianate, water-insoluble, 0.4 mg.	19	Small and medium	68	21	Medium and large
Flavianate, water-soluble, 0.4 mg.	31	Medium	101		

* 10 rat units of follutein gave 30 mg. ovaries containing a few corpora lutea.

we have so far failed to observe any loss of the potency of the follicle-stimulating preparation.

DISCUSSION

The results reported in Tables I to IV permit the following conclusions.

The non-specific augmentation of pituitary gonadotropic frac-

tions by tannic acid (Table I) differs with the preparation used. The degree of augmentation of follicle-stimulating fractions with tannic acid depends on the purity of the material; *i.e.*, a highly purified follicle-stimulating preparation will show only a slight increase of ovarian development and no marked change in ovarian weight at lower levels. The gonadotropic effect of a purified

TABLE IV

Effect of Picric and Flavianic Acids on Gonadotropic Potency of Hypophyseal Follicle-Stimulating Hormone

Preparation, total dose	Alone		Plus 10 rat units follutein*	Plus tannic acid†	
	Ovaries	Size of follicles	Ovaries (with large follicles and corpora lutea)	Ovaries	Size of follicles
	mg.		*mg.*	*mg.*	
Follicle-stimulating hormone, 8 rat units (2 mg.)	31	Large	98	73	Large‡
Picrate, water-insoluble, 2 mg.	37	"‡	76	73	"‡
Same, 1 mg.	34	"	74	45	"‡
Picrate, water-soluble, 2 mg.	43	"	109		
Same, 1 mg.	40	"	146		
Flavianate, water-insoluble, 2 mg.	24	Medium‡	68	65	Large
Same, 1 mg.	17	Large	70	43	"
Flavianate, water-soluble, 2 mg.	48	"‡	63		
Same, 1 mg.	39	"‡	96		

* 10 rat units of follutein gave 30 mg. ovaries containing a few corpora lutea.

† The amount of tannic acid was twice the weight of the protein material.

‡ Few corpora lutea present.

luteinizing (interstitial cell-stimulating) hormone preparation, on the other hand, is definitely increased by the addition of tannic acid. While 20 and 10 rat units of interstitial cell-stimulating hormone have no marked influence on the infantile ovary when administered alone, corpora lutea, follicular development, and estrous uteri are observed at these levels when given in combina-

tion with tannic acid. Addition of tannic acid will greatly enhance the activity of unfractionated pituitary extracts and of combinations of the follicle-stimulating hormones and the luteinizing hormones, as shown by increased ovarian weight and development. The observations recorded here agree with our studies on the non-specific augmentation of gonadotropic pituitary fractions observed on addition of zinc sulfate or casein (2).

As can be seen from Tables II to IV, precipitation with either picric or flavianic acid does not result in a loss of follicle-stimulating potency as claimed by Fevold, since both the water-insoluble and water-soluble fractions, when given in combination with follutein, produce marked synergism. Apparently Fevold did not avail himself of this method for determining follicle-stimulating activity. The degree of the non-specific augmentation of the picrates and flavianates with tannic acid depends on the purity of the material and is much more pronounced in the case of the follicle-stimulating hormone with a minimum effective dose of 0.25 mg. (Table IV) than in the case of a follicle-stimulating hormone with a minimum effective dose of 0.05 mg. (Table III). Since Fevold did not give the activity of his follicle-stimulating hormone preparation in units per mg., it is somewhat difficult to compare his results with our observations. We interpret the apparent reactivation which Fevold observed as being due to the addition of tannic acid, which causes the follicle-stimulating hormone to manifest itself more markedly, and not to the removal of the picric or flavianic acid. The experiments reported, therefore, do not substantiate Fevold's claim of a chemical difference between the follicle-stimulating hormone and the luteinizing hormone in regard to their reaction with picrolonic, picric, or flavianic acid, although we do not deny that there is a chemical difference between the two hormones.

SUMMARY

1. The effect of picric and flavianic acids on the potency of hypophyseal follicle-stimulating preparations has been studied.

2. The claim of Fevold that follicle-stimulating hormone is reversibly inactivated by picric or flavianic acid could not be substantiated.

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THE DETERMINATION OF VITAMIN A AND CAROTENE WITH THE PHOTOELECTRIC COLORIMETER*

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The Carr-Price reaction (1) has been employed for a considerable period of time in testing biological materials for vitamin A, and it is generally accepted that the amount of blue color produced by the reaction of vitamin A with antimony trichloride is proportional to the amount of vitamin A present. The instability of the resulting blue color, however, has definitely limited the application of the reaction for the quantitative determination of vitamin A. Recently Dann and Evelyn (2) have shown that this limitation can be overcome by the use of a direct reading photoelectric colorimeter (3).

On the basis of spectrophotometric and colorimetric readings on a series of oils and concentrates these investigators (2) obtained a factor of 0.41 ± 0.05 for converting $L_{1\,\text{cm.}}^{1\%}$ (620 mμ)[1] into $E_{1\,\text{cm.}}^{1\%}$ (328 mμ). The question of the factor to be used in converting $E_{1\,\text{cm.}}^{1\%}$ (328 mμ) into biological units of vitamin A, however, is at present unsettled. Although the factor 1600, recommended by the League of Nations Conference on Vitamin Standards (1934), has been widely used for converting $E_{1\,\text{cm.}}^{1\%}$ (328 mμ) into biological units of vitamin A, Mead, Underhill, and Coward (4) using crystalline vitamin A esters have recently arrived at a factor of 2000.

Since the factor for converting $E_{1\,\text{cm.}}^{1\%}$ (328 mμ) into biological

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[1] The term $L_{1\,\text{cm.}}^{1\%}$ (620 mμ) proposed by Dann and Evelyn (2) for use in photoelectric colorimetry is analogous to $E_{1\,\text{cm.}}^{1\%}$ (620 mμ) used in spectrophotometry.

units of vitamin A is subject to controversy, it was decided to determine directly the relationship between $L^{1\%}_{1\ \mathrm{cm.}}$ (620 mμ) and biological activity rather than use the indirect spectrophotometric basis. For this purpose the $L^{1\%}_{1\ \mathrm{cm.}}$ (620 mμ) of the U.S.P. reference cod liver oil was determined and the potency of the reference oil was checked by biological assay against β-carotene.

Since the photoelectric colorimeter also affords a rapid means of determining carotene, and it is often desirable to determine carotene and vitamin A in the same solution, the $L^{1\%}_{1\ \mathrm{cm.}}$ (440 mμ) of β-carotene was determined in chloroform and Skellysolve solutions. A correction factor was also determined for the amount of light absorbed by the blue color produced by the reaction of carotene with antimony trichloride.

Preliminary vitamin A determinations made on halibut liver oil and on the unsaponifiable matter of cod liver oil indicated that 2 − log G[2] was not a strictly linear function of the concentration of vitamin A. Similar results were obtained with β-carotene in Skellysolve and chloroform solutions. The values of 2 − log G were therefore determined over a wide range of concentrations of these chromogens.

EXPERIMENTAL

Methods

Preparation of Reagent—For the preparation of the antimony trichloride reagent, chloroform (Merck, reagent) was thoroughly washed with distilled water, dried over anhydrous potassium carbonate, and distilled in an all-glass still under reduced pressure at 30–35°, the first 25 ml. of the distillate being discarded. Antimony trichloride (Merck, reagent) was quickly added from freshly opened bottles; solution was effected by shaking the mixture at room temperature. The above procedures were carried out in the dark as much as possible. The reagent was siphoned into the reservoir of an automatic pipette which was designed to deliver 9.0 ml. of reagent in less than 2 seconds. The concentration of the reagent used in these studies was approximately 225 gm. per liter.

[2] The density of the sample solutions is expressed as 2 − log G, where G is the corrected galvanometer reading. The terms 2 − log G_{620} and 2 − log G_{440} specifically designate the density of the sample solutions when Filters 620 and 440, respectively, are used.

The chloroform used as a solvent for the samples was merely dried over potassium carbonate and filtered through sintered glass. It was protected from moisture and light at all times. The chloroform used in making up the antimony trichloride reagent was found to be unsuitable as a solvent for the vitamin A concentrates because of the rapid destruction of vitamin A by the phosgene which was invariably formed in the alcohol-free chloroform. Phosgene formation did not take place in the reagent, because the antimony trichloride exerted a stabilizing influence on the chloroform.

Peroxide-free ether was prepared by treating commercial ether with sodium bisulfite as described by Smith (5). Aldehyde-free alcohol was prepared by reduction with zinc and sodium hydroxide as described by Dubovitz (6).

Method for Determination of Vitamin A—A 0.3020 gm. sample of U.S.P. reference cod liver oil was weighed into a 50 ml. Erlenmeyer flask to which were added 10 ml. of aldehyde-free alcohol and 2.0 ml. of 60 per cent aqueous KOH; the mixture was placed on a water bath and boiled for 2 minutes. The saponified solution was cooled and transferred to a separatory funnel with 40 ml. of distilled water and 50 ml. of peroxide-free ether. The contents were thoroughly shaken and allowed to separate. The aqueous layer was drawn off into another separatory funnel and again extracted with 25 ml. of ether. The combined ether extracts were washed once with 15 ml. and twice with 35 ml. of water. The ethereal layer was dried over anhydrous sodium sulfate and decanted; the sodium sulfate was washed three times with 10 ml. of ether. The washings were combined with the ether extract in a special evaporating flask which could be simultaneously connected to a vacuum pump and a nitrogen cylinder. This was placed on a water bath at 40° and the ether was removed under reduced pressure in the presence of a gentle stream of nitrogen. The last traces of moisture were driven off by dipping the flask for several seconds in a 70° water bath. When the residue was thoroughly dry, the stop-cock leading to the vacuum pump was closed and the nitrogen stream continued until a slight positive pressure of nitrogen was obtained in the flask. The nitrogen line was then disconnected from the evaporator and chloroform was introduced. The unsaponifiable residue was diluted to a 25 ml. volume with chloroform. Eleven aliquots in duplicate of this

solution ranging from 0.15 to 1.0 ml. were transferred to absorption test-tubes by means of a micropipette, diluted to 1.0 ml. each with chloroform, and the tubes tightly stoppered. The light intensity in the colorimeter[3] was adjusted to give a galvanometer reading of 100 when a blank containing 1.0 ml. of chloroform and 9.0 ml. of antimony trichloride reagent was used. Each tube was then placed in the colorimeter and exactly 9.0 ml. of antimony trichloride reagent were added from the automatic pipette. The galvanometer was read at the point of temporary stability, Filter 620 being used. The galvanometer readings were corrected and the corresponding values of $2 - \log G$ were recorded.

Method for Determination of β-Carotene—A Skellysolve solution (Skellysolve B, redistilled, b. p. 68–70°) of S. M. A. β-carotene containing 13.00 micrograms of β-carotene per ml. was used as a stock solution in the calibration of the photoelectric colorimeter for the determination of β-carotene. Twelve aliquots in duplicate of this solution were diluted to 10 ml. with Skellysolve in volumetric flasks. The exact concentration of these solutions was determined with a universal spectrophotometer, the value 2290 being used for the extinction coefficient, $E_{1\,\text{cm.}}^{1\%}$ (475 mμ), of a Skellysolve solution of β-carotene (7). This same series of solutions was then read on the photoelectric colorimeter against a blank of pure Skellysolve, Filter 440 being used. The galvanometer readings were recorded and the values of $2 - \log G$ corresponding to the corrected galvanometer readings were obtained. In order to determine carotene in chloroform solution a measured amount of the stock solution of β-carotene in Skellysolve was evaporated to dryness in an evaporator by the method described above. The carotene was then dissolved in chloroform and diluted to the same volume. Twelve aliquots in duplicate ranging from 0.10 to 2.00 ml. were measured out and diluted to 10 ml. as before. The solutions were then read against a blank of pure chloroform with Filter 440 and the values of $2 - \log G$ were determined.

Correction for Presence of β-Carotene in Vitamin A Determina-

[3] The photoelectric colorimeter used was of the Evelyn type, manufactured by the Rubicon Company of Philadelphia. Filters 620 and 440 used in the determination of vitamin A and carotene, respectively, were the standard filters supplied with the instrument and recommended by Dann and Evelyn (2) for these determinations.

tion—A stock solution of β-carotene dissolved in chloroform containing 14.1 micrograms of carotene per ml. was used in the determination. Aliquots ranging from 0.1 to 0.7 ml. were measured out with a micropipette and diluted to 1 ml. in the absorption test-tubes. Each tube was placed in the colorimeter and 9.0 ml. of antimony trichloride reagent were added from the automatic pipette. The values of $2 - \log G$ were determined, Filter 620 being used. A tube containing 1.0 ml. of chloroform and 9.0 ml. of reagent was used for a blank.

Method of Biological Assay—The method of biological assay described by Sherman (8) was used for determining the vitamin A potency of the U.S.P. reference cod liver oil.

S. M. A. β-carotene was used for comparison in the assay of the U.S.P. reference cod liver oil. The crystals were dissolved in Skellysolve and diluted to suitable volume so that the selected daily dose of 2 micrograms of carotene, as determined spectrophotometrically, was contained in 0.2 ml. The carotene solutions were stored in the dark at 3°.

Fresh samples of U.S.P. reference cod liver oil, with an assigned vitamin A potency of 3000 U.S.P. XI units per gm., were weighed and diluted to volume with Skellysolve. The solutions used in feeding contained 5.555 gm. of the reference cod liver oil per liter of solution and were likewise stored in the refrigerator. The U.S.P. reference cod liver oil was fed in Skellysolve solution at two levels, Level A to supply 3.33 units of vitamin A based on the assigned value of 3000 units per gm. (equivalent to 2 micrograms of β-carotene), and Level B to supply 3.33 I.U. based on the apparent potency calculated from the $L^{1\%}_{1\,\text{cm.}}$ (620 mμ) determined with the photoelectric colorimeter.[4]

[4] Numerous vitamin A determinations were made with the photoelectric colorimeter on the unsaponifiable matter from two samples of U.S.P. reference cod liver oil at various concentrations. The average $L^{1\%}_{1\,\text{cm.}}$ (620 mμ) of the oil was found to be 3.45. If this value is multiplied by 0.41, the factor obtained by Dann and Evelyn for converting $L^{1\%}_{1\,\text{cm.}}$ (620 mμ) into extinction coefficients, the value of $E^{1\%}_{1\,\text{cm.}}$ (328 mμ) = 1.414 is obtained. If this figure is multiplied by 1600, the factor recommended by the League of Nations Conference on Vitamin Standards (1934) for converting $E^{1\%}_{1\,\text{cm.}}$ (328 mμ) into I.U. of vitamin A, the value of 2263 I.U. per gm. is obtained for the U.S.P. reference cod liver oil. In Level B the U.S.P. reference cod liver oil was fed to supply 3.33 I.U. calculated on this basis.

The carotene content of the solutions used in feeding was determined spectrophotometrically at biweekly intervals. The $L^{1\%}_{1\,cm.}$ (620 mμ) of the cod liver oil solutions was determined on the photoelectric colorimeter at biweekly intervals. For this purpose the solvent was removed under reduced pressure from a measured amount of the solution, and the determination was made on the unsaponifiable matter. It was found that the destruction of β-carotene at this concentration in Skellysolve solution stored at 3° in the absence of light was only about 0.5 per cent in 30 days.[5] The vitamin A content of the cod liver oil stored in the same manner in Skellysolve solution decreased 2.3 per cent in 30 days.

TABLE I

Gains of Vitamin A-Deficient Rats Receiving β-Carotene and U.S.P. Reference Cod Liver Oil

Supplement fed daily	No. of rats	Average gain in weight at end of			
		1st wk.	3rd wk.	5th wk.	7th wk.
		gm.	*gm.*	*gm.*	*gm.*
β-Carotene (2 γ)...	11	6.9	27.1	44.2	54.9
Cod liver oil, Level A*..................	6	8.2	30.5	45.5	53.9
" " " " B†........	9	9.9	32.6	53.4	68.1

* 3.33 U.S.P. units of vitamin A based on the assigned value of 3000 U.S.P. units per gm. for the U.S.P. reference cod liver oil.

† 3.33 I.U. of vitamin A based on the determined $L^{1\%}_{1\,cm.}$ (620 mμ) of 3.45 and the factors 0.41 and 1600 for converting $L^{1\%}_{1\,cm.}$ (620 mμ) into I.U. of vitamin A. See foot-note 4 to the text.

The aliquots used for feeding were corrected for any slight deterioration that was observed.

Results

Potency of U.S.P. Reference Cod Liver Oil As Determined by Biological Assay—The results of the biological assay of the U.S.P. cod liver oil are given in Table I. It is apparent that the average gain obtained when the reference oil was fed at Level A,

[5] More concentrated solutions of several samples of S. M. A. crystalline carotene and S. M. A. β-carotene in Skellysolve (400 to 500 micrograms per ml.) showed from 11 to 40 per cent destruction in 30 days storage at 3° in the dark.

to supply 3.33 U.S.P. units of vitamin A based on the assigned value of 3000 U.S.P. units per gm., is in agreement with that obtained from 2 micrograms of β-carotene (equivalent to 3.33 units of vitamin A). The average gain of the rats receiving the U.S.P. reference cod liver oil at Level B[4] was appreciably greater than that obtained with 2 micrograms of β-carotene or with the reference cod liver oil at Level A. The assigned value of 3000 U.S.P. units of vitamin A per gm. for the U.S.P. reference cod liver oil was therefore accepted for the calibration of the photoelectric colorimeter.

TABLE II

Variation of 2 − Log G with Concentration of Vitamin A

$K_1 = 45.60$.

Galvanometer reading (corrected)	$2 - \log G$	$2 - \log G$ per I.U. vitamin A	Vitamin A in test solution		Per cent error
			Actual	Calculated $(2 - \log G) K_1$	
			international units	*international units*	
91^{0}	0.0410	0.0226	1.81	1.87	+3.32
83^{0}	0.0815	0.0225	3.62	3.74	+2.76
68^{3}	0.1619	0.0224	7.25	7.38	+1.89
57^{1}	0.2422	0.0223	10.87	11.03	+1.47
47^{3}	0.321	0.0222	14.49	14.63	+0.97
40^{0}	0.398	0.0220	18.12	18.14	+0.11
34^{0}	0.471	0.0217	21.75	21.43	−1.24
28^{2}	0.543	0.0214	25.39	24.76	−2.48
24^{0}	0.620	0.0214	28.99	28.27	−2.48
21^{1}	0.683	0.0210	32.61	31.14	−4.51
17^{1}	0.760	0.0209	36.24	34.65	−4.39

Relation between 2 − Log G and Concentration of Vitamin A and Carotene—The results given in Table II show that $2 - \log G$ is not strictly a linear function of the concentration of vitamin A, but that a distinct decrease in $2 - \log G$ per I.U. of vitamin A occurred as the concentration of vitamin A was increased in the test solution. Similar results were obtained with β-carotene in chloroform and Skellysolve solutions as shown in Table III. It is evident that constants for converting $2 - \log G$ into units of vitamin A and micrograms of β-carotene are applicable only over a limited range of concentrations. Such a constant, $K_1 = 45.60$, was derived by

averaging the values obtained by dividing the concentration of vitamin A, expressed as I.U. present in the test solution, by the corresponding 2 − log G of these solutions over the galvanometer range from 30 to 70. Similar constants, $K_2 = 3.19$ and $K_3 = 2.65$, were derived for converting 2 − log G into micrograms of β-carotene per ml. in chloroform and Skellysolve solutions respectively. When these constants were applied, the $L^{1\%}_{1\,\text{cm.}}$ (440 mμ) of β-carotene was found to be 1645 and 1980 in chloroform and Skellysolve solutions respectively.

TABLE III

Variation of 2 − Log G with Concentration of β-Carotene in Chloroform and Skellysolve

2 − log G		β-Carotene			Per cent error	
			Calculated (2 − log G)K			
Chloroform	Skellysolve	Actual	Chloroform	Skellysolve	Chloroform	Skellysolve
		γ per ml.	γ per ml.	γ per ml.		
0.0434	0.0506	0.130	0.138	0.134	+5.80	+3.08
0.0848	0.0996	0.260	0.270	0.264	+3.85	+1.54
0.1264	0.1487	0.390	0.404	0.394	+3.59	+1.02
0.1659	0.1973	0.520	0.529	0.523	+1.71	+0.58
0.2460	0.2760	0.780	0.785	0.784	+0.64	+0.51
0.3300	0.3870	1.04	1.05	1.03	+0.96	−0.96
0.4010	0.478	1.30	1.28	1.27	−1.54	−2.30
0.478	0.565	1.56	1 52	1.50	−2.56	−3.84
0.542	0.636	1.82	1.73	1.68	−4.95	−7.68
0.602	0.710	2.08	1.92	1.88	−7.70	−9.62
0.663	0.770	2.34	2.12	2.04	−9.40	−12.81
0.710	0.834	2.60	2.26	2.21	−13.10	−14.99

The galvanometer range of 30 to 70 was selected because the percentage variation of 2 − log G with the galvanometer readings is less over this range than at either end of the scale.

If the concentration of chromogen in the test solution is adjusted so that a galvanometer reading between 30 and 70 is obtained, an error of less than 2 per cent is incurred by the use of constant K_1 and less than 3 per cent by the use of constants K_2 and K_3 as shown in Tables II and III. Beyond this range the error is greater, especially at the higher concentrations. If calibration curves, made by plotting concentration of vitamin A and carotene against

the corresponding values of $2 - \log G$ are used, these errors are eliminated.

The variation of $2 - \log G$ with the amount of blue color produced by the reaction of antimony trichloride with various concentrations of β-carotene is shown in Table IV. The series of dilutions used covers the range of carotene usually encountered in the determination of vitamin A in biological materials. Since the range of galvanometer readings was necessarily small, the constant $K_4 = 156.3$ was calculated for the entire range from 87^3 to 98^0 and is the average of the values obtained by dividing the number of

Table IV

Application of Correction Factor $K_4 = 156.3$ for Amount of Blue Color Produced by Reaction between β-Carotene and Antimony Trichloride

β-Carotene in test solution	Galvanometer reading (corrected)	$2 - \log G_{440}$	$2 - \log G_{620}$ Determined	$2 - \log G_{620}$ Calculated C^*/K_4	Per cent error
γ		γ *per ml.*			
1.05	98^0	0.0084	0.0088	0.0067	−23.82
1.41	97^2	0.0078	0.0110	0.0090	−18.18
2.82	95^2	0.0071	0.0200	0.0180	−10.00
4.23	93^2	0.0069	0.0292	0.0271	−7.23
5.64	91^3	0.0066	0.0374	0.0361	−3.48
7.05	90^1	0.0063	0.0446	0.0451	+1.12
8.46	88^3	0.0061	0.0518	0.0541	+4.44
9.87	87^3	0.0057	0.0568	0.0631	+11.09

* C = micrograms of β-carotene in the test solution.

micrograms of carotene in the test solution by the corresponding $2 - \log G$. The error involved by the use of this constant is given in Table IV.

Calculation of Results—The following calculations are involved if the constants given in this paper are to be used to convert the values of $2 - \log G$ into micrograms of β-carotene or I.U. of vitamin A.

$(2 - \log G_{620})K_1$ = I.U. of vitamin A in the 10 ml. test solution (uncorrected for the presence of carotene) where $K_1 = 45.60$.

$(2 - \log G_{440})K_2$ = micrograms of β-carotene per ml. in the 10 ml. test solution (chloroform) where $K_2 = 3.19$.

$(2 - \log G_{440})K_3$ = micrograms of β-carotene per ml. in the 10 ml. test solution (Skellysolve) where $K_3 = 2.65$.

$(2 - \log G_{440})K_3/K_4$ = correction factor to be subtracted from $2 - \log G_{620}$ for the amount of the blue color produced by the reaction of antimony trichloride with the β-carotene in the 10 ml. test solution where $K_4 = 156.3$. When K_3 and K_4 are combined the following equation is obtained.

$(2 - \log G_{440})K_5$ = correction factor for β-carotene where $K_5 = 0.0204$.

$[(2 - \log G_{620}) - (2 - \log G_{440})K_5]K_1$ = I.U. of vitamin A in the 10 ml. test solution (corrected for the presence of carotene).

DISCUSSION

Although the photoelectric colorimeter affords a rapid and reliable means of determining carotene and vitamin A, there are certain inherent limitations in its use. It was found that $2 - \log G$ for vitamin A and β-carotene is not a strictly linear function of the concentration of the chromogens. It is therefore apparent that constants for converting $2 - \log G$ into units of vitamin A and micrograms of carotene are not absolutely accurate. To obtain a high degree of accuracy with the instrument, a calibration curve should be used in which the values of $2 - \log G$ are plotted against the concentration over a series of dilutions of the material to be determined. By employing such a calibration curve the errors resulting from the non-linear relationship between the values of $2 - \log G$ and the concentration may be completely avoided.

When such a high degree of precision is not essential, however, conversion factors may be used without introducing serious errors provided the concentration of chromogen in the test solution is such as to give a galvanometer reading over a limited range. The constants for vitamin A and carotene given above are for solutions of such concentration as to give galvanometer readings between 30 and 70; their use results in an error of less than 3 per cent. Constants to cover a still narrower range of the galvanometer scale would produce appreciably less error.

The observation that a straight line relationship does not exist between $2 - \log G$ and concentration of vitamin A and β-carotene does not confirm the work of Dann and Evelyn (2). Shrewsbury,

Kraybill, and Withrow (9), however, using a photoelectric photometer of their own design (10), observed that density was not a linear function of the concentration of β-carotene. These workers concluded that the non-linearity was due to the relatively wide spectral band which the filters they used transmitted. Filter 440 used in the work reported in the present paper had a transmission band of from 410 to 475 mμ and Filter 620 had a transmission band of from 595 to 660 mμ. It is apparent that an instrument employing a filter with a transmission band of 65 mμ will not give the degree of linearity which can be obtained with a spectrophotometer in which a spectral band of only a few mμ can be selected to correspond exactly to an absorption maximum of the compound.

On the basis of the results of the biological assays and the chemical determinations of vitamin A made on the U.S.P. reference cod liver oil and the factor 0.41 of Dann and Evelyn (2), a factor of 2120 was derived for the conversion of $E_{1\,\text{cm.}}^{1\%}$ (328 mμ) into I.U. of vitamin A. This is in close agreement with the value of 2150 given by Mead, Underhill, and Coward (4) as a result of their work with vitamin A-2-naphthoate. The figure 1440 given by Dann and Evelyn (2) for the $L_{1\,\text{cm.}}^{1\%}$ (440 mμ) of β-carotene is somewhat lower than the value given in this paper. They state, however, that their value was undoubtedly too low because of the impurity of the β-carotene used. Although the S. M. A. β-carotene used in the work reported here was not absolutely pure, the exact concentration of β-carotene in the solutions was determined spectrophotometrically. It is believed, therefore, that $L_{1\,\text{cm.}}^{1\%}$ (440 mμ) = 1645 is near the correct value for β-carotene in chloroform solution.

SUMMARY

1. The U.S.P. reference cod liver oil when assayed biologically in comparison with β-carotene was found to have the assigned potency of 3000 I.U. of vitamin A per gm. This same oil was found to have an average $L_{1\,\text{cm.}}^{1\%}$ (620 mμ) of 3.45 as determined with the Evelyn photoelectric colorimeter.

2. Calculations are presented for converting colorimeter readings into I.U. of vitamin A and micrograms of carotene. Constant $K_1 = 45.60$ was derived for converting $2 - \log G_{620}$ into I.U. of vitamin A, and constants $K_2 = 3.19$ and $K_3 = 2.65$ were derived

for converting $2 - \log G_{440}$ into micrograms of β-carotene in chloroform and Skellysolve solutions respectively. A correction factor was also derived for the light absorbed by the blue color produced in the reaction between β-carotene and antimony trichloride.

3. It was found that $2 - \log G$ was not a strictly linear function of concentration of vitamin A or carotene. The constants given were calculated for concentrations giving galvanometer readings from 30 to 70. If these constants are used within this range, an error of less than 3 per cent is incurred. For more accurate work it is recommended that a calibration curve be used in which $2 - \log G$ is plotted against concentration of the chromogen.

4. β-Carotene was found to have an average $L_{1\,\text{cm.}}^{1\%}$ (440 mμ) of 1645 and 1980 in chloroform and Skellysolve solutions respectively.

5. On the basis of the biological assays, the determined $L_{1\,\text{cm.}}^{1\%}$ (620 mμ) of 3.45 for the U.S.P. reference cod liver oil, and factor 0.41 for converting $L_{1\,\text{cm.}}^{1\%}$ (620 mμ) into $E_{1\,\text{cm.}}^{1\%}$ (328 mμ), a factor of 2120 was obtained for converting $E_{1\,\text{cm.}}^{1\%}$ (328 mμ) into I.U. of vitamin A per gm.

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ESSENTIAL FATTY ACIDS, VITAMIN B_6, AND OTHER FACTORS IN THE CURE OF RAT ACRODYNIA*

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Dermal abnormalities in the rat have been recognized as having a dual origin; *viz.*, from an "essential" fatty acid deficiency (Burr and Burr, 1929), or a vitamin B_6 deficiency (György, 1935). That some relationship exists between these two deficiencies was suggested by the finding of Birch and György (1936) that certain fats cured the dermatitis as produced by vitamin B_6 deficiency. The point was emphasized when Quackenbush, Platz, and Steenbock (1939) showed that linoleic acid, curative in "essential fatty acid deficiency," was also curative for acrodynia. Birch (1938) examined the relation of vitamin B_6 and the fatty acids of corn oil in the cure of acrodynia and concluded that at least two factors were operative, vitamin B_6 and a second factor present in corn fatty acids. This was based on the observation that a vitamin B_6 preparation failed to cure rat acrodynia unless the fatty acids from corn oil were fed as well. However, Quackenbush, Platz, and Steenbock (1939) had shown that the fatty acids from corn oil cured acrodynia when fed alone and hence apparently no additional vitamin B_6 was needed. This suggested that either (1) fatty acid preparations from corn oil contained vitamin B_6 in addition to the second factor postulated by Birch or (2) corn oil fatty acids were capable of supplanting vitamin B_6 plus any other factor necessary in the cure of acrodynia. It was the purpose of this investigation to examine these possibilities and to study further

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the relationship between acrodynia and the Burr and Burr syndrome.

EXPERIMENTAL

Acrodynia was produced on a fat-free, vitamin B_6-deficient diet as follows: Weanling rats, 35 to 40 gm., as prepared by the technique of Quackenbush, Platz, and Steenbock (1939) were placed on Diet V. This diet consisted of a mixture of cerelose (commercial glucose) 78, casein (alcohol-extracted) 18, Wesson's salts (1932) 4, supplemented daily with 10 micrograms of thiamine and 20 micrograms of riboflavin dissolved in 1 drop of 0.02 N acetic acid, and 10 micrograms of carotene and 5 micrograms of calciferol dissolved in 1 drop of liquid hydrogenated coconut oil. On this diet the rats developed an acute acrodynia in 4 to 5 weeks. Materials were tested for antiacrodynic potency by feeding them as supplements to Diet V while the animal was continued on the diet. If a cure resulted in 3 weeks of such supplementation and was maintained for an additional 3 weeks, the material was regarded as potent. The reliability of this curative test for antiacrodynic potency has been demonstrated with more than 500 rats. A material once shown to be potent was consistently confirmed in its potency when subjected to retest. Spontaneous cures were never observed.

For the study of the antiacrodynic potency of fats two oils were selected as examples; *viz.*, corn oil (Mazola) and cottonseed oil (Wesson oil). These oils were curative at levels of approximately 5 and 10 mg. per day. If this curative action was dependent upon a content of vitamin B_6, then the removal of the vitamin, if it existed in the fat, should have resulted in a loss of activity of the fat. Although at this point in our investigation the basic, water-soluble properties of vitamin B_6 did not strengthen the view that fats contained the vitamin, Kuhn and Wendt (1938) demonstrated that vitamin B_6 contained two alcoholic and one phenolic group which made ester combinations at least a possibility. Although Keresztesy and Stevens (1938) showed that vitamin B_6 was unaffected by alkali and hence could presumably survive saponification, it was difficult to understand how the vitamin could appear in fatty acid preparations such as were fed by Birch. These doubts were confirmed by an extended series of

experiments which will not be detailed here. Suffice it to say that when corn or cottonseed oil was used as a source of active fat, continuous ether extraction of fatty acid soaps, continuous extraction of fat with dilute acid, refluxing with dilute acid, electrodialysis of the saponification mixture of an active fat, and the use of adsorbing agents in ethereal solutions of fat, all failed to diminish the antiacrodynic potency of the fatty acids. That vitamin B_6 was not part of the antiacrodynic potency of fats seemed certain after the following experiments were performed.

Kuhn and Wendt (1938) had shown that a methyl ether of vitamin B_6 was formed by treatment with diazomethane. According to Möller (1938) the methyl ether of vitamin B_6 is biologically inactive. This suggested the possibility of preparing methyl esters of antiacrodynic fatty acids which would be free of active vitamin B_6 by treating the crude fatty acids with diazomethane in ether solution. If, for some obscure reason, vitamin B_6 had been brought into the solution of the fatty acids, treatment with diazomethane would have resulted in the formation of the methyl ether. Then, if the potency of the fatty acids was indeed dependent upon a content of vitamin B_6, there would be a resultant loss in antiacrodynic activity. However, esters of corn and cottonseed oil fatty acids (fed to four and two rats respectively), as well as the ester of linoleic acid (fed to two rats), prepared by the action of diazomethane on the free acids were all curative within 3 weeks when 20 mg. of the preparation were administered daily.

That the antiacrodynic activity of fatty acids was not due to vitamin B_6 *per se* was further confirmed by the demonstration that the antiacrodynic activity of fatty acids from corn oil was retained after three successive precipitations with barium in alkaline solution. 25 gm. of corn oil were saponified with alcoholic KOH and the soaps poured into a saturated solution of $Ba(OH)_2$. The barium soaps were filtered off, and the acids regenerated with HCl and extracted with ether. The ether was removed under reduced pressure and the potassium and barium soaps formed again as before. This was repeated again and the fatty acids were finally esterified with ethyl alcohol by refluxing with sulfuric acid. The esters were active in curing acrodynia in two rats given 20 mg. daily. Cure was effected within 3 weeks. Similar

precipitation of the acids of cottonseed oil and of linoleic acid resulted in active ester preparations which when administered to three and two rats respectively at the rate of 20 mg. daily cured acrodynia within 3 weeks. As vitamin B_6 itself does not form an insoluble barium salt, the conclusion seemed inescapable that vitamin B_6 was in no way involved in the cure of acrodynia when it was effected by certain fats.

The curative properties of the fatty acids of corn and cottonseed oil were thus again referred to the fatty acid fraction of which linoleic acid had been demonstrated as a potent member. Since linoleic acid, as well as arachidonic (Turpeinen, 1938) and linolenic acids, is prepared from active oils, the possibility has been existent that their activity was dependent upon the inclusion of the true active material. Synthesis of these materials would eliminate these objections but as yet no practicable syntheses have been devised. It was thought desirable, therefore, to examine an active oil, corn oil, for constituents which might form a part of the fatty acid fraction as ordinarily prepared and have antiacrodynic activity, and yet not be an unsaturated fatty acid of the linoleic, linolenic, or arachidonic acid series. Two such types of compounds were investigated; *viz.*, phenols and certain lactones. The phenolic compounds in corn oil (Mazola) were obtained as follows: 1 kilo of corn oil was saponified, the unsaponifiable compounds were removed by ether extraction, and the potassium soaps poured into an excess of $Ba(OH)_2$ solution. The Ba soaps were filtered off and the Ba removed from the filtrate by H_2SO_4. The acidified filtrate was then extracted with ether and the combined ether extracts washed, dried with Na_2SO_4, and the ether removed under reduced pressure. Approximately 400 mg. of residue remained, insoluble in water but soluble in 5 per cent Na_2CO_3. Addition of CO_2 to saturation permitted the ether extraction of materials which gave an intense blue color with Folin's phenol reagent. When the material was fed, it was found devoid of antiacrodynic activity (Table I).

Another group of compounds which might be found in fatty acid fractions from oils is composed of certain lactones which are reformed on acidification of the saponification mixture, such as the coumarins. True fatty acids are separable from such compounds by extraction from lipid solution with aqueous K_2CO_3.

Possible lactones present in corn, cottonseed, and wheat germ oil were prepared for feeding in the following manner. The fat was saponified with alcoholic KOH, diluted, and the non-saponifiable fraction removed by petroleum ether extraction. The solution was then acidified and the fatty acids removed with petroleum ether, washed, and the fatty acids extracted from the petroleum ether solution with successive portions of 5 per cent K_2CO_3. The K_2CO_3 was then washed out with water and the petroleum ether solution was dried with Na_2SO_4. The solvent was removed under reduced pressure. The residue was taken up in liquid hydrogenated coconut oil for feeding. Test (Liebermann-Burchard) showed that the residues contained some sterols which

TABLE I

Antiacrodynic Potency of Crude Phenols and Lactones Present in Antiacrodynic Oils

Preparation	Amount fed daily	Original oil equivalency	Effect on acrodynia (3 wks.) (4 rats)
		mg.	
1. Crude phenols of corn oil	0.4 mg.	1000	Negative
2. " lactones " " "	140 γ	40	"
K_2CO_3-extracted acids from (2)..	20 mg.	20	Curative
3. Crude lactones of cottonseed oil.	90 γ	40	Negative
K_2CO_3-extracted acids from (3)	20 mg.	20	Curative
4. Crude lactones of wheat germ oil.	240 γ	40	Negative
K_2CO_3-extracted acids from (4).	20 mg.	20	Curative

were incompletely removed with the non-saponifiable fraction. Yields of residue per gm. of fat were corn oil 3.5, cottonseed oil 2.3, and wheat germ oil 6.0 mg. When these residues were fed, they proved to be totally inactive, while the fatty acids which had been removed by the K_2CO_3 extraction proved active when fed as the ethyl esters (Table I).

The preceding experiments thus redemonstrated that the antiacrodynic activity of certain vegetable oils was resident in the fatty acid fraction as a fatty acid. These fatty acids had usually been fed as the ethyl esters as prepared by refluxing the acids with anhydrous ethyl alcohol and sulfuric acid. Formation of the methyl esters from the free acids by diazomethane had left

the antiacrodynic activity unimpaired. It was thought that some clue as to the chemical nature of the active fatty acid might be obtained, other than that it was probably unsaturated, by preparing the ethyl or methyl esters of the active fatty acids in other ways. Therefore, methyl esters of fatty acids from corn and cottonseed oils were prepared by formation of the silver soaps and refluxing with methyl iodide. The resulting esters were active (Table II). The ethyl esters of the same acids were also active when prepared from the sodium salt by refluxing with diethyl sulfate (Table II).

Quackenbush, Platz, and Steenbock (1939) showed that elaidinization of linoleic acid resulted in a loss of antiacrodynic potency. It appeared desirable to determine whether another type of molecular alteration, hydrogenation, would destroy the activity of

TABLE II

Esters of Antiacrodynic Fatty Acids As Prepared by Different Esterifying Agents Curative within 3 Weeks

Preparation (20 mg. fed daily)	No. of rats	Iodine value (Hanus)
Methyl esters of corn oil acids by methyl iodide......	2	117.6
" " " cottonseed oil " " "	2	96.0
Ethyl " " corn oil by diethyl sulfate...........	5	115.0
" " " cottonseed oil by diethyl sulfate.. .	5	98.0

linoleic acid. Consequently a previously assayed preparation of ethyl linolate (iodine value = 159) was hydrogenated over platinum. Hydrogen absorption was complete in ½ hour. The ethyl stearate thus formed (iodine value = 0) was tested for antiacrodynic potency on five rats at 4 times the original curative level and failed to cure.

Water-Soluble Factors in Cure of Acrodynia—It seemed fairly well established by the above that the antiacrodynic potency of certain fats was resident in the unsaturated fatty acids of which linoleic acid was representative. To determine under what conditions vitamin B_6 could effect a cure of rat acrodynia was the object of the succeeding experiments.

Birch (1938) failed to cure rat acrodynia on a fat-free diet when

he fed a vitamin B_6 preparation obtained by alcoholic extraction of yeast. However, in experiments similar to those of Birch, we have been able to cure rat acrodynia with another source of water-soluble B vitamins. When rats exhibiting acrodynia as produced in this laboratory on Diet V were fed 100 mg. of a rice bran concentrate (vitab[1]) per day, the acrodynia was cured in

TABLE III

Antiacrodynic Potency of Water-Soluble Factors of Rice Bran Concentrate (Vitab)

Preparation	Vitab equivalent fed daily	No. of rats	Effect on acrodynia (3 wks.)
	mg.		
1. Rice bran concentrate	100	12	Curative
	50	2	Negative
1,*a*. " " " (ether-extracted)	100-150	4	Curative
2. Fullers' earth filtrate from (1)	150	11	Negative
	200	5	"
3. " " eluate from (1)	600	9	Temporary improvement; relapsed
4. Combined filtrate and eluate		4	Curative
	γ		
5. Crystalline vitamin B_6	10	12	Temporary improvement; relapsed
	15	2	" "
	25	1	" "
	50	1	" "
	mg.		
6. Filtrate factor (2) fed with 10 γ vitamin B_6 to rats having relapsed on 10 γ vitamin B_6	150	4	Curative

3 weeks (Table III). The vitab was completely water-soluble and when diluted formed a clear solution. A diluted sample of vitab was extracted by three shakings with ether (pH = 4.85)

[1] Vitab, Type II, is a commercial B vitamin concentrate prepared from rice bran. It was supplied by Vitab Products, Inc., Emeryville, California.

and concentrated *in vacuo* to its original volume without any loss in potency (Table III).

Since a similarity between "essential fatty acid" deficiency and acrodynia had already been indicated by the curative effects of fats and linoleic acid in both deficiencies, it appeared desirable to determine whether this similarity could be extended to include curative effects of the two deficiencies by the same water-soluble concentrate, ether-extracted vitab. Since in the production of the Burr and Burr syndrome the B vitamins have been fed in a limited amount of yeast (0.7 gm. daily) in comparison with the acrodynia-producing Diet V which supplies merely thiamine and riboflavin, it was possible to study the effect of yeast vitamins on the production of dermal symptoms by progressively increasing the amounts of dried yeast. Accordingly sixteen weanling rats, prepared as usual, were divided into four groups of four. Diet V was modified to include 1, 2, 4, and 8 per cent yeast at the expense of the glucose and was fed to Groups I, II, III, and IV respectively. Carotene, calciferol, thiamine, and riboflavin were fed in addition as usual. Each group was continued on its respective diet until growth stopped and the animals remained stationary or lost weight for a continuous period of 3 weeks. Weight records were kept and the appearance of symptoms noted. When the growth curves had remained at a plateau for 3 weeks, two animals of each group were fed 22 mg. of vacuum-distilled ethyl linolate prepared via the tetrabromides from corn oil by the method of Rollet (1909). The remaining two rats in each group were then fed 100 mg. daily of ether-extracted vitab. The supplementation was continued for a period of 6 weeks during which increase in weight was noted as well as the disappearance of dermal symptoms.

The growth of the animals on the various yeast levels is indicated in Fig. 1. The cessation of growth of the rats receiving 8 per cent yeast at approximately 175 gm. of body weight is similar to that observed by Turpeinen (1938) in a recent study of the essential fatty acids. Like Turpeinen we observed only mild dermal symptoms in the animals receiving these large amounts of yeast. Scaliness of the paws and tail was the chief symptom. At successively lower levels of yeast, however, this mild scaliness was observed to pass into more severe types until at 1 per cent

yeast the dermatitis was so severe, involving the paws, mouth, eyes, and ears, as to be indistinguishable from the acute acrodynia

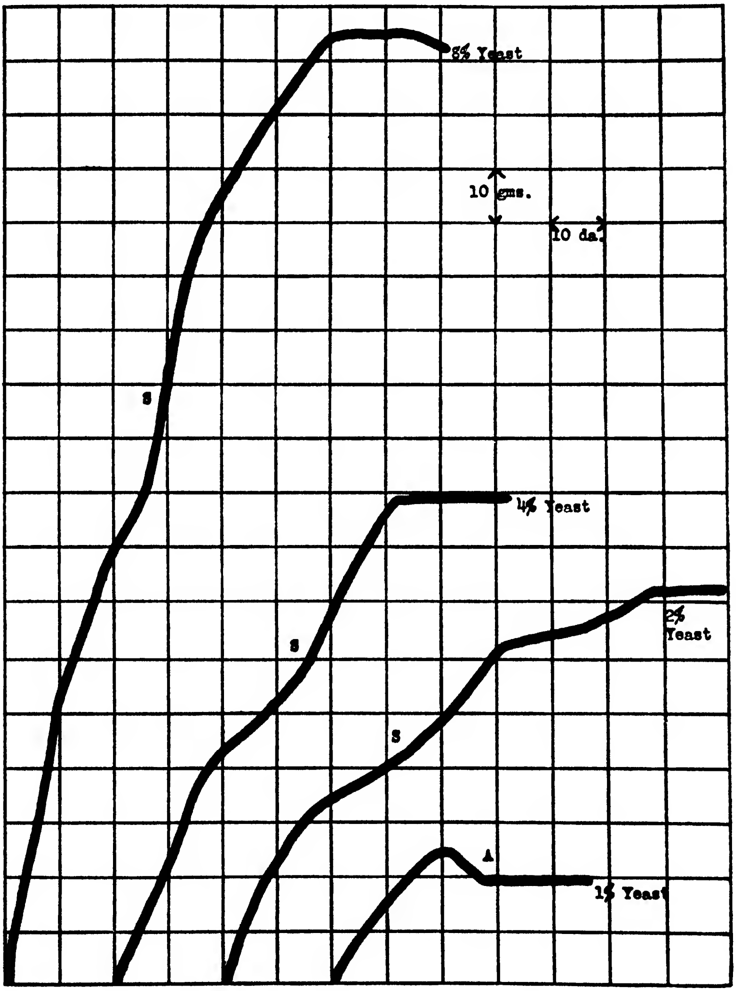

Fig. 1. Growth of rats on low fat diet (Diet V) containing varied amounts of dried brewers' yeast. S = scaliness; A = acrodynia.

produced on Diet V. These results parallel those obtained by Birch (1938) who fed graded amounts of an alcoholic extract of

yeast. The action of yeast vitamins in the production of the Burr and Burr syndrome seems to be one of modification of the acute acrodynia into the typical scaliness.

The connection between acrodynia and the Burr and Burr syndrome was further emphasized by the parallel curative effects of both ethyl linolate and ether-extracted vitab. In the 6 weeks of feeding of these materials all dermatitis symptoms disappeared within the first 3 weeks, and in some cases even earlier (Fig. 2).

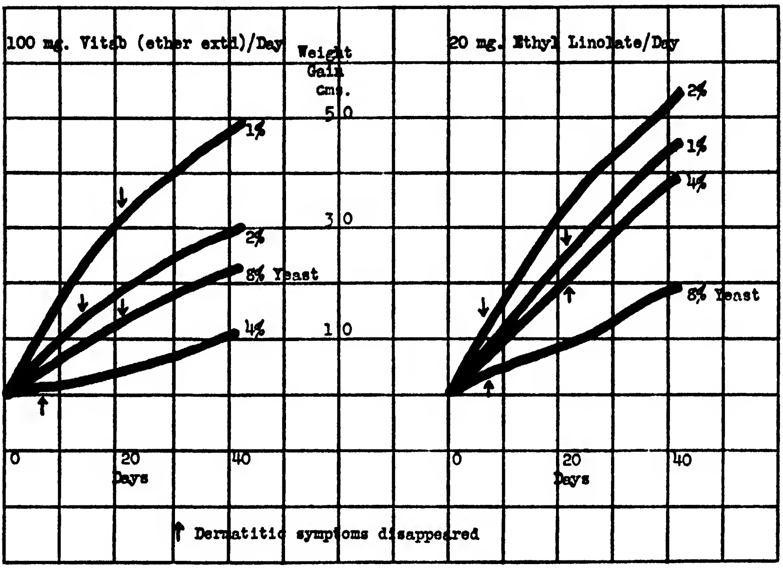

FIG. 2. Growth response to ethyl linolate and ether-extracted vitab of rats that reached a plateau on a low fat diet (Diet V) containing varied amounts of dried brewers' yeast. Each curve represents two rats.

Ethyl linolate and vitab were equally effective in restoring growth, the increments achieved on the 8 per cent yeast level being comparable to the growth obtained by Turpeinen and used by him as a criterion of "essential" fatty acid activity. Increments of growth on the lower levels of yeast were even greater.

With the demonstration that the Burr and Burr syndrome and acrodynia could both be cured by the same agents, it became possible to confine the study of the antidermatitis activity of vitab

to curative studies in acrodynia. Results could thus be obtained in a shorter time and the study much facilitated. It soon became apparent that the antidermatitis potency of vitab was in part dependent upon its vitamin B_6 content. Thus when vitab was diluted with water and shaken with five successive portions of English fullers' earth (pH 4.8), the filtrate had lost its ability to cure acrodynia (Table III). The eluate of the fullers' earth, as prepared by $Ba(OH)_2$ elution, removal of Ba with H_2SO_4, and concentration, was inactive as well. However, when the filtrate and eluate were fed together, antiacrodynic potency was observed once more (Table III). The failure of the fullers' earth eluate (vitamin B_6) to cure acrodynia on a fat-free diet thus confirmed Birch's finding. However, the curative action of vitamin B_6 became possible by the addition of a "filtrate factor." Thus the second factor of Birch is found in a water-soluble form. In fact this second factor which is necessary for the cure of acrodynia has not been shown to exist in fats at all, since originally the presence of this factor in fats was based by Birch on the ability to cure when vitamin B_6 alone had failed. This curative property of fat we have shown to be independent of vitamin B_6 and capable of curing completely in its absence. Thus while Birch's contention that a factor in addition to vitamin B_6 is needed for the cure of acrodynia is confirmed, this second factor is shown to be part of the so called filtrate factor. The importance of filtrate factor in the cure of acrodynia was indicated by György (1938) when he determined the effect of the crystalline vitamin B_6 in the cure of acrodynia. He reported that, "Even the skin effect was not regularly attained unless a further supplement corresponding to the so-called 'filtrate factor' was added."

That vitamin B_6 was indeed incapable of curing acrodynia in the absence of filtrate factor was confirmed when the crystalline vitamin[2] was fed. Although Dimick and Schreffler (1939) had shown that in the presence of a filtrate factor (likewise prepared from a rice bran concentrate) 10 micrograms of vitamin B_6 fulfilled the daily requirement of the rat for this vitamin, when 10 to 50 micrograms of the crystalline vitamin were fed to rats ex-

[2] Crystalline vitamin B_6 as isolated from rice bran concentrate was kindly furnished by Dr. S. Lepkovsky.

hibiting acrodynia on Diet V a temporary cure resulted seldom lasting longer than 2 weeks. It was followed by a relapse into a florid dermatitis as acute as that originally observed and the animals declined and died without ever showing subsequent improvement during the remaining period of vitamin B_6 feeding (Table III).

A few studies have been made of the chemical nature of the factor in the fullers' earth filtrate which is necessary in conjunction with vitamin B_6 for a cure of acrodynia. Continuous extraction of diluted vitab (pH 4.8) with ether in a Kutscher-Steudel apparatus for 48 hours did not diminish the antiacrodynic potency, nor did continuous extraction with ether diminish its potency after it had first been made alkaline with NaOH (pH 8.5). $Ba(OH)_2$ was added to vitab to alkalinity (phenolphthalein) and allowed to stand overnight. Precipitated material was removed by filtration and the barium removed from the filtrate by H_2SO_4. Concentration to the original volume resulted in a preparation which had the complete antiacrodynic potency of the original vitab. The second factor is thus not precipitable by barium.

Since the essential fatty acids are capable of curing rat acrodynia unaided, it might be assumed that the true antiacrodynic substance must be closely related to these acids, possibly as a metabolic product. Since the cyclic, N-containing vitamin B_6 seems far removed from such a type of compound, it would appear that possibly it is the "accessory factor" which is more directly concerned with the cure of acrodynia. Vitamin B_6 would thus be concerned with the conversion of the inactive "accessory factor" into the active, true antiacrodynic substance.

We desire to express our appreciation of the assistance of Mr. Fred A. Kummerow in making some of the preparations.

SUMMARY

Acrodynia, as it is known in the rat, can be cured by two different means.

1. It can be cured by the so called "essential fatty acids." This action is independent of vitamin B_6, since "essential fatty acid" preparations have been shown not to contain any vitamin B_6 and to retain their activity after treatment with diazomethane.

2. It can be cured by rice bran concentrate. This action is independent of fatty acids, but is dependent upon vitamin B_6 plus a second "accessory factor." This second factor has been shown to be included in the filtrate from the fullers' earth treatment of rice bran concentrate.

Vitamin B_6, both in crude preparation and in crystalline form, had only a temporary effect on acrodynia in the absence of the "accessory factor."

Like acrodynia, the Burr and Burr syndrome was cured by both "essential fatty acids" and rice bran concentrate. The Burr and Burr syndrome was produced by the addition of dried brewers' yeast to our acrodynia-producing diet. Gradations between the severe symptoms of acrodynia and the mild symptoms of the Burr and Burr syndrome were obtained by feeding increasing amounts of yeast.

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A MICRO BLOOD ESTERASE DETERMINATION APPLIED TO STUDIES OF RATS BEARING ADENOCARCINOMA

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The esterase activity in blood or tissues has usually been measured by titration of the acid liberated in the hydrolysis of the ester used as substrate. Cattaneo and Scoz (1) in 1937 determined esterase activity by an electrometric titration, using the null point method, with quinhydrone electrodes. Their method required the use of 1 cc. of serum, and measured the hydrolysis of tributyrin in a buffer mixture of ammonia and ammonium chloride of pH 8.

Green in 1934 (2), using a titration method requiring 0.5 cc of serum, reported that during the growth of Jensen sarcoma in the rat the esterase content of the serum of the rats fell progressively. Because of the large amount of serum required it was necessary to sacrifice the rat for each determination, and the change in enzyme activity with development of the tumor could not be followed on the same rat at progressive stages of tumor growth. Consequently there were exceptions in the attempt to correlate the tumor growth with enzyme activity.

In the experiment described below, a micromethod was devised by which the blood esterase activity of an individual rat could be measured at frequent intervals. Wistar rats were used, three-fourths of which bore implanted adenocarcinomas. The decrease in pH of a buffered solution, due to the butyric acid liberated from the hydrolysis of ethyl butyrate by rat blood, was taken as a measure of the blood esterase.

Method

To 1 cc. of M/15 phosphate buffer solution (pH 8.9) in a serological test-tube, was added 0.05 cc. of oxalated rat blood obtained

from the tail. Buffer solution was drawn twice through the micropipette used for the blood, in order to rinse it. 1 drop of ethyl butyrate, purified as indicated below, was introduced. The mixture was then well shaken by a rotary motion, and a paraffined cork inserted. After incubation of the tube for exactly 3 hours in a water bath at 38° ± 0.1°, it was immediately cooled to room temperature and the pH of the contents determined by means of a quinhydrone electrode. The difference between the pH of this solution and that of a blank run without blood was taken as a measure of the esterase activity. Duplicate deter-

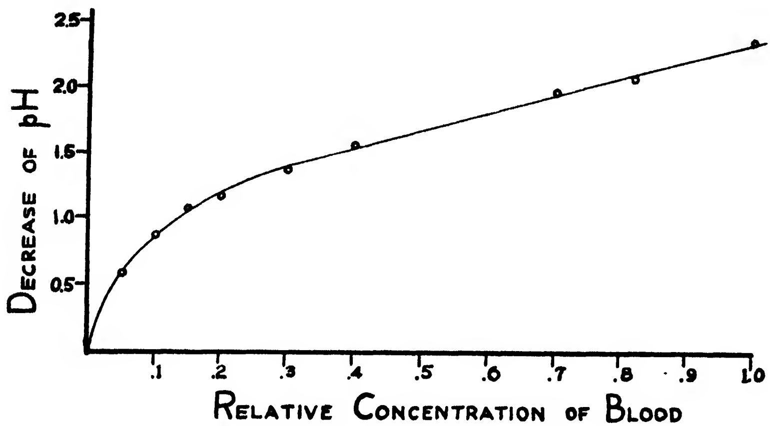

FIG. 1. Decrease of pH caused by varying amounts of esterase, obtained by decreasing the concentration of a single sample of normal rat blood.

minations on the same blood sample usually checked to within 0.02 pH, with a maximum observed variation of 0.05 pH.

Results obtained on blanks run with the addition of enzyme inactivated by heating in a water bath at 60° for 1 hour were compared with the results obtained on blanks run with the blood omitted. No differences could be detected, and it was therefore concluded that the addition of the small amount of oxalated blood used had no appreciable effect on the pH of the buffer solution. The pH of the blank as measured on the glass electrode remained constant at 8.41 throughout the experiment.

The relationship between concentration of enzyme and pH change obtained is shown in Fig. 1. The data for this curve

were obtained by use of a series of dilutions of the blood of one normal rat.

The ethyl butyrate used was a purified fraction prepared from a technical grade. The technical ester was treated with a solution

TABLE I

Measurements of Tumors at Various Intervals after Inoculation of Peritoneal Fluid Containing Adenocarcinoma Cells

Rat No.	Size of tumor 10 days after inoculation	Days after inoculation	Size of tumor	Remarks
	cm.		*cm.*	
1	3.2 × 2.1	16	3.8 × 2.1	Necrosis, killed 16th day
2	2.5 × 1.3	16	3 × 1.6	" " 16th "
3	2.5 × 1.9	16	2.5 × 2.5	Killed 16th day
4	1.3 × 1.3	16	1.9 × 1.9	Healed to a scab*
5	1.8 × 2.8			Killed 11th day
6	2.1 × 1.6	16	2.5 × 3.2	†
7	1.3 × 1.0	14	1.6 × 1.9	‡
8	2.2 × 1.3	14	2.8 × 2.5	Killed 14th day
11	2.8 × 1.6	16	3.3 × 3.2	Necrosis, killed 16th day
12	3.5 × 1.9	16	4.4 × 3.2	Killed 16th day
13	2 8 × 1.6	16	4.4 × 3.2	§
14	2.5 × 1.9	14	3.2 × 2.5	‖
15	3.2 × 1.3	14	4.1 × 2.2	¶
16	3.5 × 2.2	16	4.4 × 3.2	Necrosis, killed 16th day
17	2.5 × 2.5	16	3.2 × 3.2	" very anemic, killed 16th day

* Rat 4 had completely recovered on the 26th day.

† Rat 6 had a slightly smaller tumor with a necrotic center on the 21st day; the rat had to be sacrificed for use in further implantations.

‡ Rat 7 showed a receding tumor; on the 17th day the tumor was 1.6 × 1.6 cm.; on the 26th day only a slight lump and scar remained.

§ Rat 13 showed a necrotic tumor with size unchanged on the 21st day.

‖ Rat 14 was killed on the 17th day; the tumor was 4.6 × 2.5 cm. with necrosis.

¶ Rat 15 was killed on the 17th day; the tumor was 4.3 × 3.0 cm. with necrosis.

of sodium carbonate and then washed with water. It was dried, first over anhydrous potassium carbonate, and then over P_2O_5. The neutral, dry ester was fractionally distilled three times and the fraction boiling between 118–123° collected for use. As the ester is somewhat unstable in the presence of traces of mois-

ture, it was stored in a flask having two fused-in side arms with stop-cocks, to each of which was attached a sealed P_2O_5 tube. One of the side arms was a fine capillary tube so arranged as to serve as a siphon for obtaining a portion of the ester. When an aliquot was being obtained for analysis, the ester was kept anhydrous by admitting air through an open P_2O_5 tube, and the capillary siphon tube was filled with ester from which drops could be delivered to the reaction tube. Variation in the size of the drop did not affect the accuracy of the method, as 1 drop of the

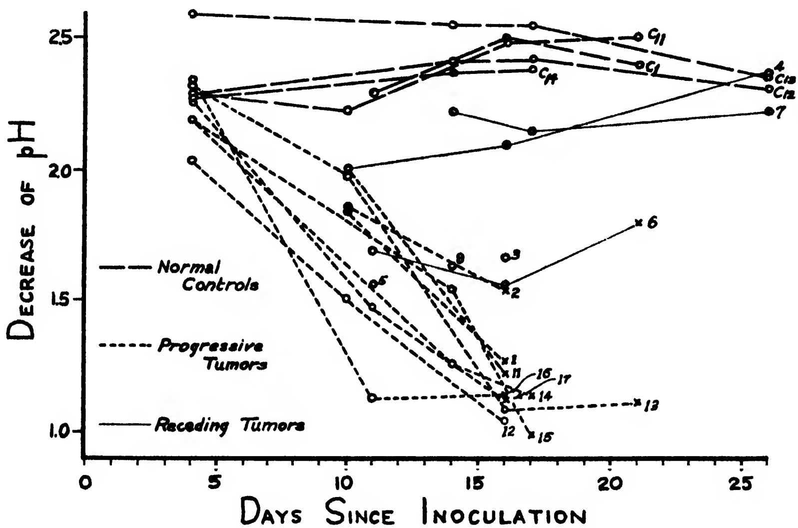

FIG. 2. Effect of progressing and receding tumors on the esterase content of the blood of rats with adenocarcinoma. The figures on the curves refer to the rat numbers. X = necrosis.

neutral ester was more than enough to saturate the test solution used, and the determination was shown to be independent of the amount of excess beyond saturation.

EXPERIMENTAL

Two litters of the same age (37 days) were used simultaneously. Litter 1 contained nine rats, of which one male (Rat C_1) was left as an uninoculated control. Litter 2 contained eleven rats; of these, three females and one male (Rats C_{11}, C_{12}, C_{14}, and C_{13}) were

left uninoculated. Eight rats from Litter 1 and seven rats from Litter 2 were inoculated in the groin with 0.5 cc. of peritoneal fluid containing adenocarcinoma cells. The peritoneal fluid was obtained from a rat about 2 months old, into whose peritoneal cavity several small, non-necrotic pieces of adenocarcinoma had been implanted 10 days previously.

All of the animals inoculated developed growing tumors. The measurements of these tumors at various time intervals after inoculation are given in Table I. Determinations of esterase activity were made on tail blood at various intervals up to 26 days after inoculation, as shown in Fig. 2. The rats were killed at the time intervals indicated in Table I. More than one determination was made on all rats except three (Nos. 3, 5, and 8). The results obtained on these three rats were consistent with the results on the other rats.

The results shown in Fig. 2 when compared with the growth of tumor given in Table I indicate that, during the growth of an implanted adenocarcinoma, the esterase activity of the blood of rats falls far below the range of fluctuations shown by normal rats, and that there is a progressive decrease with the development of the tumor in a given rat. The results on Rats 4, 6, and 7 show that the presence of a receding tumor is accompanied by a rising of the blood esterase activity toward normal values.

SUMMARY

A micro esterase determination was devised, so that changes in the blood esterase activity of a single rat could be followed during the growth of an implanted adenocarcinoma. During the growth of the tumor, the esterase activity of the blood fell far below the range of fluctuation of blood esterase values found for normal rats. Three of the rats exhibited receding tumors; in each case a blood esterase activity rising toward normal values accompanied the recession of the tumor.

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THE FORMATION OF CREATINE FROM GLYCOCYAMINE IN THE LIVER

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The study of the precursors of creatine in animals has been beset by two difficulties principally. One has been the lack of really adequate biological material; the other, the lack of a specific, and at the same time sensitive analytical method. Experiments hitherto have consisted in attempts to change the urinary excretion of creatine and creatinine, or the creatine content of the tissues of intact animals or of isolated perfused organs. The normal, *i.e.* uncontrolled, fluctuations in tissue composition and urinary excretion are relatively large compared with the changes induced experimentally; it is often impossible to distinguish when experimental effects are observed, whether these have arisen from changes in the processes of excretion or synthesis; there may be variations in the water content of the tissues, thereby affecting their percentile composition; all of these have stood in the way of firm conclusions being drawn.

Some of these difficulties have been avoided in perfusion experiments on isolated organs (1). These experiments are extremely laborious, time-consuming, and costly. The experimentally induced change in creatine content is at the most—about 30 per cent—a small deviation from the normal. The normal base-line is not constant but varies with the age and weight of the animal. Since the same specimen cannot be used for the experiment and control, a large number of animals must be used first to establish the normal base-line, encompassing the variations of the normal, and then an equally large number for each single experiment in order to obtain a body of data sufficiently large for a statistical conclusion which may be significant.

In 1935 we published some observations of a slight increase in in "apparent" creatine when rat liver slices were incubated with a protein hydrolysate (2). Granted that such liver slices could synthesize creatine, most of the difficulties in working with whole animals or whole organs are avoided. With tissue slices the one specimen of tissue provides material for controls and for testing a variety of experimental variants simultaneously. This was the reason that the observation of even a slight synthesis of creatine by tissue slices seemed promising.

We were faced here, however, with the second difficulty mentioned above, uncertainty whether the material which is augmented by the liver slices is really creatine. A number of substances give a positive test with the Jaffe reagent. The amount of the material in question formed by the liver slices was too small to be identified by isolation.

Accordingly further study of this problem was postponed until a more specific analytical method which could be adapted to submicro scale was available. Such a method was provided by Dubos and Miller (3). They discovered and succeeded in culturing a soil bacterium which specifically destroys creatine and creatinine.

We again took up the problem, employing tissue slices and this new adjuvant to our former submicromethod for creatine (4). We have found that liver slices of the cat, rabbit, and rat are able to convert glycocyamine to creatine. In the experimental conditions we have observed, the increase is 5 to 20 times the amount originally present in the slices. The difficulties and uncertainties which exist in conclusions resting on statistical analysis of small differences have therefore been overcome. The liver is well suited to this study, because its normal creatine content and therefore the control or blank value is very low.

We have found further that when methionine is present in the Ringer's solution, the amount of creatine formed from the added glycocyamine was on the average about 50 per cent greater than when methionine was not added. We have tested thirty-two other substances including amino acids, methylated amines and a purine, and betaine. All of these were negative in this respect. It seems reasonable to conclude therefore that rat liver slices transfer the methyl group of methionine to glycocyamine, thus converting the latter substance to creatine.

Technique

The tissue slice technique used is only slightly modified from that described by Warburg (5). The details of the reaction vessels and the manner in which they are mounted are described in a previous communication (6).

The blank controls and each experimental variant were carried through in triplicate. We have had twenty to thirty reaction vessels, each containing three slices from the same liver, running simultaneously. It was necessary to make the sampling of the slices as uniform as possible, because the capacity to synthesize creatine may vary significantly between the lobes. The procedure we finally adopted was to arrange the reaction vessels in three rows (when each experimental variant is carried through in triplicate), one vessel in each row for each variant. Consecutive slices were transferred to a Petri dish containing Ringer's solution, one slice for each vessel in the row. After 3 minutes soaking in this vessel, with gentle agitation, the slices were transferred to the reaction vessels. The same was done for the other two rows. The whole procedure was then repeated according to the number of slices wanted in each vessel. The reason for the 3 minutes soaking in the Ringer's solution before transfer to the reaction vessels is that more creatine was formed than when the slices were transferred directly with only momentary rinsing in Ringer's solution.

At the end of the reaction period the contents of the vessels, including the slices, were transferred to test-tubes, and the bottles and transfer pipette washed with two 1 ml. portions of 0.02 N HCl. The test-tubes were placed in a boiling water bath for 10 minutes with occasional stirring or shaking. After this time the test-tubes were cooled and the volumes made up to 6 ml. with 0.02 N HCl. These test-tubes are marked at 6 ml. Usually only 0.1 to 0.2 ml. was needed to bring the volumes to the mark. The suspensions were then filtered. The coagulated slices with the coagulated protein in each vessel were transferred to small glass dishes and the weight determined after drying at 100°.

The analytical procedure was essentially that described previously (4). Some minor details were varied from time to time in addition to employment of the bacterial digestion procedure of Dubos and Miller. Table I is a protocol of an experiment in

which the complete analytical procedure was used. Instead of triplicate reaction vessels, six were used for each experimental variant. The boiled extracts in pairs of vessels were joined and filtered. To 8 ml. of this filtrate were added 2 ml. of 0.25 M phosphate buffer at pH 7.0. This solution was divided into two portions. In one-half the creatine was determined directly. The creatine in the other half was destroyed by a suspension of NC soil organisms prepared according to the prescription of Dubos and Miller. The difference between the color developed by the Jaffe reagent with and without this digestion gave the true creatine (and creatinine) in the original solution.

The bacterial digestion was carried out as follows: 5 ml. of the solution neutralized with phosphate were transferred to a 250 ml. Erlenmeyer flask. To this was added 1 ml. of a suspension of the NC soil organisms. The necks of the flasks were covered with squares of Parafilm and set away in an air bath at 38° for $\frac{1}{2}$ to $\frac{3}{4}$ of an hour. At the end of this time 1 ml. of 0.5 N HCl was added to each flask. The contents were then centrifuged. 6 ml. of the clear supernatant solution were taken for analysis. The length of time the bacteria were allowed to react on the experimental solutions was based on a prior determination of the potency of the bacterial suspension used. It was the time required by 1 ml. of the bacterial suspension to destroy completely the creatine in 5 ml. of a 2 mg. per cent solution. This was a larger amount of creatine than in any of the tissue extracts submitted to digestion.

To the 5 ml. of tissue extract containing the phosphate buffer, but which had not been digested by the bacteria, were added 1 ml. of water and 1 ml. of 0.5 N HCl. 6 ml. were then taken for analysis. From this point on the analytical procedure was identical for the solutions which had been digested with bacteria and those which had not. Both sets were carried through to the completion of the analysis simultaneously.

The 6 ml. aliquots were transferred to thick walled Pyrex test-tubes with internal dimensions of 125 × 12 mm. The tubes were covered with parchment paper caps and autoclaved for 20 minutes at 125°. After they were cooled, a small amount of Lloyd's reagent was added to each. We have found that the amount of the Lloyd's reagent may vary from 10 to 60 mg. without affecting the final result. The test-tubes were now shaken for 7 minutes

on a shaker of the type devised by Fisher and Wilhelmi (7). The tubes were then centrifuged, the supernatant solution discarded with the last drop at the rim of the test-tube taken up with filter paper, and the Lloyd's reagent resuspended in 2 ml. of 0.01 N HCl. The tubes were centrifuged again, the supernatant solution again discarded, and the adherent moisture on the walls of the test-tube carefully taken up with filter paper. 3 ml. of a sodium picrate solution were added to each tube. This solution consists of 10 parts of saturated picric acid (purified) and 1 part of 10 per cent NaOH, these being mixed immediately before use. The test-tubes were again shaken for 7 minutes and centrifuged. The color was measured on a spectrophotometer with light of approximately 0.525 μ wave-length. With concentrations ranging from 0 to 2 mg. per cent there is a strictly linear relation between concentration of creatine and the intensity of color measured in this manner.

The following controls were taken through the above identical procedure including digestion by the bacteria: Ringer's solution alone, Ringer's solution containing the same concentration of glycocyamine used in the experiment, and Ringer's solution containing glycocyamine and methionine. Each of these was carried out in triplicate. In addition 1 ml. of the bacterial suspension alone and bacterial suspension plus 5 ml. of 2 mg. per cent creatine were incubated with the experimental solutions and carried through the same analytical procedure, performed in duplicate. Finally a set of five standard creatine solutions with concentrations ranging from 0.1 to 2 mg. per cent was treated in exactly the same manner as the experimental solutions except that they were not submitted to bacterial digestion. The amounts of creatine in the experimental solutions and controls were determined by interpolation from the straight line given by the readings of these standard solutions. The above controls and the standards were carried through afresh in every experiment with the experimental solutions.

It is a testimony to the convenience of this analytical method that we have frequently carried through more than 60 individual analyses from the bacterial digestion to the final spectrophotometer reading in less than 5 hours.

We found in experiments with rat and rabbit liver that essen-

TABLE I

Formation of Creatine by Rat Liver Slices in 6 Hours at 37.5° from Glycocyamine

Treatment	Composition of medium				Apparent creatine				Residual chromogenic material after bacterial digestion	True creatine			
	Ringer's solution	Glycocyamine	*dl*-Methionine	Dry weight of tissue	Total	Total amount formed	Amount formed from glycocyamine	Q(creatine) × 100 from glycocyamine		Amount present	Total amount formed	Amount formed from glycocyamine	Q(creatine) × 100 from glycocyamine
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
	ml.	*mg.*	*mg.*	*mg.*	*mg. per cent*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
Analyzed immediately	4			40.8	0.12				0.07	0.05			
" "	4			41.5	0.14				0.07	0.07			
" "	4			59.8	0.17				0.10	0.07			
Incubated 6 hrs.	4			34.1	0.21	0.12			0.14	0.07	0.03		(0.15)
" 6 "	4			40.2	0.26	0.14			0.18	0.08	0.03		(0.13)
" 6 "	4			38.0	0.22	0.12			0.14	0.08	0.03		(0.15)
" 6 "	4	0.25		35.4	1.20	0.60	0.38	1.9	0.70	0.50	0.45	0.42	2.1
" 6 "	4	0.25		45.0	1.54	0.94	0.66	2.5	0.72	0.82	0.77	0.74	2.8
" 6 "	4	0.25		35.5	1.31	0.71	0.49	2.4	0.72	0.59	0.54	0.51	2.5
" 6 "	4	0.25	1.45	32.5	1.54	0.90	0.70	3.7	0.72	0.82	0.78	0.75	3.9
" 6 "	4	0.25	1.45	37.8	1.54	0.90	0.64	2.9	0.70	0.84	0.79	0.76	3.4
" 6 "	4	0.25	1.45	35.5	1.53	0.89	0.67	3.2	0.71	0.82	0.77	0.74	3.6
" 6 "	4	0.25			0.61				0.61				
" 6 "	4	0.25			0.60				0.60				

Incubated 6 hrs.	4	0.25			0.60				0.61				
" 6 "	4	0.25	1.45		0.65				0.64				
" 6 "	4	0.25	1.45		0.66				0.63				
" 6 "	4	0.25	1.45		0.61				0.64				
1 ml. bacterial suspension incubated 45 min. at 37°									0.01 0.007				
0.1 mg. creatine incubated with 1 ml. bacterial suspension 45 min. at 37°									0.006 0.006				

The factor converting mg. per cent to Q(creatine) is $4/100 \times 1.5 \times 171 \times 1/6 \times 1/$(dry weight of tissue): 4/100, since there were only 4 ml. in each reaction vessel for the amount of tissue indicated; 1.5 because these 4 ml. were diluted to 6 ml. in the transfer from the reaction vessels to the test-tubes in which they were boiled; 171, because 1 mg. of creatine would occupy 171 c.mm. at S.T.P. if it were a gas; 1/6, because the experiment was run for 6 hours. Q(creatine) is therefore the amount of creatine formed, expressed as if it were a gas in c.mm. at S.T.P., per mg. of tissue (dry weight) per hour.

tially the same result was obtained whether bacteria were used or not. Accordingly we later dispensed with the use of the bacteria, except as indicated.

Results

Table I is a condensed protocol of a typical experiment. The figures in Column 6 are the spectrophotometer readings converted to mg. per cent by interpolation from the standard curve. The figures in Column 7 are obtained by subtracting from those in Column 6 the values of the glycocyamine or glycocyamine plus the methionine blank, and the amount present in the tissue at zero time. The glycocyamine blank value is quite large. It arises from the conversion of glycocyamine to glycocyamidine during the autoclaving. It was essential that the glycocyamine blanks be treated exactly the same as the experimental solutions through all the operations from immersion in the water bath at 37.5° for the same length of time to the final development of the color. The figures in Column 8 are obtained by subtracting from those in Column 7 the amount of chromogenic material formed by the tissue in the Ringer's solution without glycocyamine. *Q*(creatine) (Columns 9 and 14) is the amount of creatine formed, expressed as if it were a gas in c.mm. at S.T.P., per mg. of tissue (dry weight) per hour. The figures in Column 11 are the differences between those in Columns 6 and 10. From the figures for the blanks in Columns 6 and 10, it is seen that the bacteria digested none of the glycocyamine. The figures in Columns 9 and 14 are not significantly different. They are a little higher in Column 14 than in Column 9 because in Column 7 a correction should have been applied to the glycocyamine blank value for the glycocyamine converted to creatine, from 10 to 16 per cent. If this had been done, the figures in Column 8 would have been increased by 0.06, which would have made them the same, within experimental error, as those in Column 13.

In a previous determination we have found that there was practically no creatinine in the tissue extracts.

Table I shows that the liver slices convert glycocyamine to creatine. This, as far as we know, is the first time the biological conversion of glycocyamine to creatine has been demonstrated by an unequivocally specific analytical method for creatine, and in which

the tissue used both in the controls and in the experiment came from the identical organ specimen. The increases in the experimental vessels were from 10 to 15 times the amount present in the tissue at the beginning of the experiment. The figures in Columns 9 and 14 show that with added methionine there was 40 per cent more creatine formed than when methionine was not added.

Most of the chromogenic material in the liver slices at the beginning and end of the experiment was not true creatine. This is in accord with the findings of Baker and Miller (8). There is, however, a slight increase in true creatine in the liver slices suspended in Ringer's solution containing no glycocyamine. We have found this repeatedly. Most of this non-creatine chromogenic material found in the tissue blanks is also not glycocyamine. This was ascertained by a direct determination for glycocyamine.

The values for *Q*(creatine) are much smaller than those found with liver slices for the formation of urea, amino acids, or hippuric acid (6). Nevertheless, this rate, small though it is, is more than sufficient to account for the total creatine plus creatinine excretion in the rat. Thus, an adult rat with a liver weighing 12 gm. might excrete 9 mg. of creatine plus creatinine in 24 hours (9). A *Q*(creatine) of 0.02 would correspond in such an animal to the formation of 7 mg. of creatine in 24 hours.

The following compounds and combinations of compounds were tried instead of glycocyamine to determine whether they could serve as precursors of creatine: arginine, arginine plus glycine, arginine plus glycolic acid, choline, glycine, glycine plus urea, glycolic acid, and methionine. Each of these has been tested on both rat and rabbit liver slices several times. The results have been consistently negative.

Table II is a summary of most of our experiments with glycocyamine and methionine. In the course of these experiments we have used glycocyamine from two different commercial sources, and two specimens of *dl*-methionine, one prepared in this laboratory and one obtained commercially. A different animal was used for each pair of figures.

The data in Table II show the increase in creatine formation invariably obtained when methionine was added to the glycocyamine. Approximately the same relative increase occurred regardless of age, sex, and nutritional condition of the animal, and

TABLE II

Formation of Creatine by Rat Liver from Glycocyamine with and without Added Methionine at 37.5°

From glycocyamine alone	From glycocyamine and methionine	Duration of incubation	Age and sex	Nutritional condition
Q(creatine) × 100	Q(creatine) × 100	hrs.		
3.2	6.2	1	Adult. ♂	Normal nutrition
6.1	9.0	2	" ♂	" "
4.0	5.5	3	" ♂	" "
4.0	6.5	3	" ♂	" "
5.7	8.1	3	" ♂	" "
1.9	3.4	3	3 mos. ♂	" "
4.2	7.4	3	3 " ♂	" "
6.6	7.9	3	3 " ♀	" "
6.5	9.0	3	3 " ♀	" "
4.6	6.6	4	Adult. ♂	" "
8.5	11.3	4	" ♂	" "
6.1	8.5	4	" ♂	" "
7.3	10.6	4	" ♂	" "
7.7	11.1	4	" ♂	" "
3.1	5.8	4	1 mo. ♂	" "
2.3	4.5	4	1 " ♂	" "
1.5	2.3	4	Adult. ♂	Fasted 66 hrs.
1.5	2.4	4	" ♂	" 66 "
4.1	6.4	4	" ♀	Normal nutrition
4.4	6.9	4	" ♀	" "
2.7*		6	" ♂	" "
2.8*	4.8*	6	" ♂	" "
2.5*	3.6*	6	" ♂	" "
2.4	3.3	6	" ♂	" "
3.1		6	" ♂	" "
2.5		6	2 mos. ♂	" "
2.2		6	Adult. ♀	" "
2.7		6	" ♀	" "

Each of the above figures is the average of a triplicate determination. Each reaction vessel contained 20 to 40 mg. (dry weight) of liver, 4 ml. of Ringer's solution containing 0.25 mg. of glycocyamine, and in the methionine series in addition 1.49 mg. of the amino acid.

* Values obtained with the employment of bacteria in the analysis.

regardless also of the duration of the experiment. The over-all average value for Q(creatine) × 100 from glycocyamine alone is 4.1, and from glycocyamine plus methionine 6.6.

We have no explanation as yet for the large variations in the rate of creatine formation in different experiments.

It will be noted that most of the results in Table II were obtained without the employment of bacteria in the analysis. Three experiments with rat liver and three with rabbit liver were carried out in which bacteria were used. The values for Q(creatine) were, as in Table I, essentially the same with and without the use of bacteria. We feel therefore that the results obtained without the use of bacteria with this tissue and under these experimental conditions are as reliable indices of true creatine as those obtained with bacteria.

Table III

Rate for Formation of Creatine from Glycocyamine with and without Added Methionine

Time	Without methionine		With methionine	
	Amount of creatine formed per 100 mg. (dry weight) of tissue	Q(creatine) × 100	Amount of creatine formed per 100 mg. (dry weight) of tissue	Q(creatine) × 100
hrs.	*mg.*		*mg.*	
1	0.018	2.8	0.036	6.2
2	0.070	6.0	0.101	8.6
4	0.095	4.6	0.150	6.4
6	0.144	4.5	0.203	5.8

Each of these figures is the average of a triplicate determination. The composition of the Ringer's solution and amount of tissue were the same as in the experiments of Tables I and II. Two lobes of liver were used. Consecutive slices were placed alternately in the vessels with and without methionine. The comparison at each hour, therefore, is of the activity of immediately adjacent sections of liver. Bacteria were not employed in the analyses here.

Table III contains the results in more detail of an experiment in which the rate of creatine formation from glycocyamine, with and without methionine, was studied. The figures show that the methylation of glycocyamine proceeds unchecked for at least 6 hours. The maximum at 2 hours in each series is accidental. It did not occur in other similar experiments.

The absolute amount of glycocyamine methylated was not increased by a 10-fold increase in the initial concentration of gly-

cocyamine. We have not yet explored thoroughly the effect of changing the concentration of methionine. In one experiment 0.0025 M methionine was as effective as 0.01 M. The fact that addition of so much methionine increases the rate of methylation only 40 or 50 per cent leads us to suspect that the methylating agent in the liver itself may not be methionine. Another piece of evidence which points in this direction is that the ratio of the rates of creatine formation with and without methionine is nearly the same throughout the whole period of from 1 to 6 hours (Table III). In other words, the effects of the methionine and of the methylating agent in the tissues were additive. It is possible that the methylating agent in the tissues is derived from methionine.

The following compounds were tested with rat liver as possible methylating agents of glycocyamine. All gave negative results: acetylcholine, *d*-alanine, *d*-arginine, *l*-asparagine, *l*-aspartic acid, betaine, caffeine, choline, *l*-cysteine, *l*-cystine, ethanolamine, *d*-glutamic acid, *d*-glutamine, glycine, glycolic acid, guanidine, *l*-histidine, *dl*-isoleucine, *l*-leucine, *d*-lysine, mono-, di-, tri-, and tetramethylamine, *d*-ornithine, *l*-hydroxproline, *dl*-phenylalanine, *l*-proline, *dl*-serine, *d*-threonine, *l*-tryptophane, and *l*-tyrosine. The final concentration of glycocyamine in the Ringer's solution in these experiments was always approximately 0.0005 M, and 0.0025 M of the compound whose methylating possibilities were being tested. The significance of the positive effect invariably obtained with methionine is heightened obviously by the fact that all of the above compounds were negative.

Some experiments have been made with the kidneys of the cat, rabbit, and rat. Slices of the cortex with and without methionine either failed to methylate glycocyamine or the slight positive results were within the experimental error. These experiments are part of a survey not yet completed of the organs of a number of animals. The details of these experiments will be published later.

Minced liver of the rat or rabbit failed to give any measurable increase in creatine in 6 hours at 37.5° on incubation with glycocyamine, with or without methionine.

Similarly negative results were obtained with slices of heart

and sartorius. But the cell structure is not preserved in slices of these muscular structures as it is in slices of liver.

Until conditions are discovered in which positive results are obtained with minced liver, we feel that no significance can be attached to negative results with sections whose cell structure has been broken or to extracts of other organs.

DISCUSSION

Beard, Boggess, and Pizzolato (10) proposed that glycine and urea condense to form glycocyamine, which is then methylated by more glycine or glutamic acid. We have observed neither this condensation nor the proposed methylating reaction with rat or rabbit liver slices. The conclusions of Beard *et al.* are based largely on experiments on the rat. The negative results in our experiments with glycine and urea we believe are significant in view of the positive results with glycocyamine and methionine. Experiments on the synthesis of urea, amino acids, and hippuric acid have shown that results obtained with tissue slices afford reliable qualitative information, at least, regarding the potentialities of the intact tissue *in situ*.

Bodansky (11) fed glycocyamine to normal rats and at 3, 6, 12, and 24 hours after feeding measured the glycocyamine and creatine concentrations in the liver, muscle, heart, and kidney. Bodansky's interpretation of the data obtained in these experiments was that significant increases in creatine content occurred only in the kidney, and that the increases in the liver were not sufficiently clear cut to be significant. Bodansky concluded that his findings suggest "that methylation of the guanidoacetic acid may have occurred in the kidneys... In view of the occurrence of guanidoacetic acid in large amounts in the liver and the failure to show an increase in creatine, it is surmised that the liver plays an insignificant rôle, if any, in creatine production."

This surmise is in direct conflict with our observations. Bodansky's experimental observations, however, and ours are not in conflict. Thus the creatine content of the liver in two controls in Bodansky's experiments was 16.0 and 20.4 mg. per 100 gm. of tissue, and in three experiments with glycine, 18.6, 16.8, and 19.0 mg. After glycocyamine feeding the figures are 21.7 mg. in 3 hours

and 23.3 in 6 hours, and in a second experiment 30.5 in 3 hours and 22.3 in 6 hours. The relative increases over the control values in the liver with glycocyamine were of the same order of magnitude as those found in kidney. The increases in true creatine in the liver were probably relatively much larger, since most of the chromogenic material in the liver with the Jaffe reagent is not creatine (Table I, and also Baker and Miller).

There is another physiological factor which must be taken into account in interpreting the data obtained by Bodansky. This factor is that the kidney is better able to store creatine than the liver. The analyses of Baker and Miller and our own show that the true creatine content of the kidney is 4 or 5 times that of the liver. Bodansky found that when creatine was fed the creatine content of the liver was twice the control value at the 3rd hour but had declined to the control value by the 6th hour, whereas in the kidney the concentration was 70 per cent above the control value at the end of the 6th hour.

Our observations show conclusively that glycocyamine can be methylated by rat liver. The rate of methylation by kidney slices is much slower than in liver, if it is not absolutely negative.

All these observations are brought into accord by the hypothesis that in the experiments of Bodansky the creatine synthesized from glycocyamine in liver was quickly removed by the blood and stored for a relatively long period in the kidney. We have no reliable data of our own at present on the possible conversion of glycocyamine to creatine in other organs or in the muscles.

Fisher and Wilhelmi found that when isolated male rabbit heart was perfused there was an increase in creatine when arginine was added to the perfusate. No increase in creatine was observed under these conditions in the hearts of prepubertal animals. Davenport, Fisher, and Wilhelmi (12), extending these observations, found that glycolic acid was essential for the methylation of glycocyamine. They suggested the following mechanism of creatine formation in the rabbit heart. Arginine is broken down to glycocyamine and glycolic acid; the glycolic acid then methylates the glycocyamine to form creatine.

In rat and rabbit liver slices the results with arginine, with and without glycolic acid or glycine, and with the two acids alone did

not yield detectable amounts of either glycocyamine or creatine. The only substance we have yet found which is capable of methylating glycocyamine is methionine.

We have attempted to repeat the observations of Fisher *et al.* with slices of rabbit heart. These experiments were unsatisfactory because of the difficulties of obtaining uniform sampling and because the experimental effects were small compared with the amount of creatine initially present. For the reason stated above we do not attach any significance to these experiments.

The difference between the observations on the perfused heart and ours on the liver stand, for the time being at least, either as an unresolved discrepancy or as indicating important differences in the mechanism of creatine formation in the heart and in the liver.

SUMMARY

1. Liver slices of cat, rabbit, and rat convert glycocyamine to creatine.

2. This methylation is accelerated in rat liver by methionine, (other animals are now being studied).

3. Methionine is the only substance we have yet found among a large number of amino acids, methylated amines, and other compounds which is able to effect this methylation in rat liver.

The authors wish to thank Mr. Y. Tajima for the assistance he gave them throughout this work, and Dr. H. W. Davenport for advice and assistance in the construction of the shaker used here. They are indebted to Dr. R. Dubos, and wish to thank him for specimens of the bacteria used in these experiments and valuable information on the culture details.

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DIPHTHERIAL INTOXICATION AND VITAMIN C CONTENT OF THE SUPRARENALS OF GUINEA PIGS

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The finding of Harde (1) that the vitamin C content of the suprarenals of guinea pigs that died from diphtherial intoxication is reduced has been confirmed in several reports (2–6). These reports, together with the observation that under certain circumstances animals survive otherwise lethal doses of diphtheria toxin when the toxin is administered with large amounts of neutral sodium ascorbate (1, 7), have led to the theory (7–10) of a direct reaction between this substance and the toxin. No such reaction could, however, be demonstrated *in vitro* by the means available (11).

The possibility that in the body vitamin C combines directly with diphtheria toxin was investigated by injecting sublethal doses of the toxin into a number of animals and determining the vitamin C content of the suprarenals of representatives of the group at frequent intervals thereafter. In a preliminary experiment (6), the average vitamin C content of these glands was found to be increased over that of the uninjected controls examined at the beginning of the experiment. This study has been continued and certain factors that were not controlled in the preliminary experiments have been investigated.

Methods of Investigation

Diet of Test Animals—Guinea pigs were obtained from the laboratory farm and those selected for each experiment were of uniform age and dietary history. All but one of the experiments were performed during the winter months when the animals had

been receiving alfalfa hay, oats, barley, commercial rabbit pellets, cabbage, carrots, and mangels, with sodium chloride and clean water *ad libitum*. In the one experiment performed in May the animals had received supplements of fresh grass for several weeks previously.

At the beginning of each experiment the guinea pigs to be used were transferred to the "test animal quarters" where a ration similar to that on which the animals are raised is routinely fed, except that it contains no hay. In four of the experiments, however, the animals on transfer were fed only the oats, barley, and rabbit pellets which contain negligible quantities of ascorbic acid. After a preliminary period on this diet to reduce the vitamin C concentration of their tissues, each guinea pig was given a daily supplement of ascorbic acid by subcutaneous injection for the period prior to the administration of diphtheria toxin.

Weight of Animals—All the guinea pigs that were used without preliminary preparation and the majority of those that received the injections of vitamin C weighed from 230 to 280 gm. The few animals whose weight did not increase to this range during the period of treatment were divided equally between the test and the control groups at the time the toxin was injected.

Preparation of Vitamin C Solutions—The vitamin C solutions were prepared by weighing out the total dose of synthetic crystals for the daily injection of all the animals, adding the required amount of 2.089 N sodium hydroxide (1 ml. neutralizes 500 mg. of ascorbic acid), diluting to a convenient volume with 0.85 per cent sodium chloride solution, and checking the final pH (6.88 to 7.01) with a glass electrode. While the diluent and the glassware were sterilized before use, the final material was not, because ascorbic acid is heat-labile at a hydrogen ion concentration approximating that of the tissues. No inflammatory reactions suggestive of bacterial contamination were observed in any of the control animals after as many as eighteen daily injections of such material.

Diphtheria Toxin—The test animals were injected with varying amounts of diphtheria toxin No. 496B, a stable toxin used in the intracutaneous test for susceptibility to diphtheria in persons and restandardized frequently. Dilutions were prepared in

0.85 per cent sodium chloride solution immediately before injection. The majority of the animals were injected subcutaneously with a uniform sublethal dose of toxin; that is, the maximum amount which they would survive under favorable conditions. While this dose was rarely fatal to guinea pigs weighing from 230 to 280 gm., two of seventeen animals injected with it in February, 1938, succumbed in slightly more than 2 days. Eight of the animals in this experiment had already been sacrificed; otherwise it is possible that the mortality might have been higher, since at that time of year the susceptibility of guinea pigs is increased.

In a second experiment animals were injected with 1.5 times this sublethal dose. While they were sacrificed within 3 days, others in our experience when given a similar amount of this same toxin usually succumb within 10 days. Ten additional guinea pigs were injected with 3 times the standard sublethal dose of toxin and all died in from 20 to 55 hours with an average survival time of 39.3 hours.

Vitamin C Assay of Suprarenals—All animals injected with the largest dose of toxin were allowed to die from its effects. Those injected with less than this amount were sacrificed. The guinea pigs that died were autopsied within 18 hours. They were preserved in the cold during the interval. Animals that were sacrificed were autopsied immediately.

Both suprarenals were removed, weighed, and ground in a mortar with clean sand. The resultant pulp was taken up in an 8 per cent solution of trichloroacetic acid which contained 2 per cent of the metaphosphoric acid. The suspensions were cleared by centrifugation and after the residues had been washed twice with the acid mixture the pooled supernatants were titrated with 0.005 per cent 2,6-dichlorophenol indophenol solution after the method of Bessey and King (12). A stock supply of a 0.05 per cent solution of the dye was prepared each week in a buffer of pH 6.8 and stored in the cold. For the titrations a 10-fold dilution was made and standardized daily against synthetic ascorbic acid crystals.

Statistical Analysis of Results—The means and the standard deviations of the means were calculated by the customary methods. The significance of the difference between the mean

of the vitamin C content of the suprarenals of the test animals and of the controls was estimated by Fisher's t test (13).[1]

EXPERIMENTAL

In November, 1937, twenty-five guinea pigs were injected with a uniform sublethal dose of diphtheria toxin. Five animals were destroyed and their suprarenals examined for vitamin C on each of the first 3 days and on the 5th and 9th days thereafter. A similar number of uninjected animals were sacrificed and examined at the same time. The results are presented in Table I. No significant differences were found between the means of the vitamin C content of the suprarenals of the test animals and those of the controls. A progressive increase occurred, however, in the mean vitamin C content of the suprarenals of both groups throughout the period of observation, similar to that previously recorded (6). The cause of this increase of more than 100 per cent in the average vitamin C content of the suprarenals of guinea pigs trans-

TABLE I

Vitamin C Content of Suprarenals of Guinea Pigs Injected with Sublethal Dose of Diphtheria Toxin*

Days after injection of toxin	No. of animals		Vitamin C in test animals		Vitamin C in control animals		Difference between test animals and controls		
	Test	Control	Mean	Standard deviation of mean	Mean	Standard deviation of mean	Difference of means	t	Nearest tabular value P
			mg.	*mg.*	*mg.*	*mg.*	*mg.*		
1	5	5	0.042	0.009	0.048	0.009	−0.006	0.473	0.6
2	5	5	0.049	0.008	0.061	0.010	−0.012	0.965	0.3
3	5	3†	0.065	0.006	0.062	0.022	+0.003	0.140	0.9
5	4†	5	0.071	0.018	0.076	0.011	−0.005	0.231	0.8
9	5	5	0.086	0.016	0.095	0.023	−0.009	0.311	0.8

* Received routine test animal diet.

† Number reduced owing to breakage of tubes in centrifuge.

[1] P, as given in the text and tables, is the probability of similarly obtaining under the influence of a conventional normal variation a value of t numerically as great as that observed in the given case (13). A difference between means, thus tested, may be called *significant* if $P < 0.05$, and *highly significant* if $P < 0.01$.

ferred from the stock to the test animal quarters, despite the fact that they received the same sources of vitamin C in both places, is not apparent.

This observation, however, raised a question as to the interpretation of the results obtained previously in animals injected with lethal doses of diphtheria toxin. Since such animals have a pronounced anorexia prior to death, the marked decrease observed (1–6) in the ascorbic acid content of their suprarenals might have been influenced to a considerable degree by this failure to eat. Accordingly, in a second experiment begun in December, 1937, and finished in January, 1938, thirty guinea pigs of uniform weight

TABLE II

Vitamin C Content of Suprarenals of Guinea Pigs Injected with Lethal Dose of Diphtheria Toxin*

Dose†	No. of animals	Mean content of vitamin C	Standard deviation of mean	Difference of means	t	Value of P	Deviation from uninjected control
		mg.	*mg.*	*mg.*			*per cent*
0	7	0.086	0.009				
1.5	9	0.054	0.006	−0.032	2.855	0.015	−37.2
3	10	0.018	0.003	−0.068	8.489	<0.01	−79.1

* The animals were deprived of vitamin C for 7 days, and then given daily subcutaneous injections of 10 mg. of ascorbic acid for another week prior to injection of the toxin.

† Multiples of the sublethal dose used in the other experiments.

were placed on a vitamin C-deficient diet for 1 week. Four of the animals died. At the end of the 7 days the deficient diet was supplemented with daily subcutaneous injections of 10 mg. of ascorbic acid. These supplements were continued until the death of the animals. 2 weeks after the experiment was begun ten of the group were injected with 3 times the sublethal dose of the same lot of diphtheria toxin used in the previous experiment and nine others with 1.5 times as much. As each of the animals in the first group died, one in the second group, in so far as possible, and a control that had received no toxin were sacrificed and the suprarenals of the three examined at the same time. The results are given in Table II. The mean of the vitamin C content of the

suprarenals of the group that was given the smaller dose of toxin was 37 per cent less than that of the control group. The t test indicates that the difference between these two means was significant ($P < 0.05$). The decrease (79 per cent) of the same factor in the group of guinea pigs that received 3 times the uniform sublethal dose was highly significant ($P < 0.01$).

In the next experiment, forty-five animals were prepared as in the second test. Six of them died prior to the injection of the toxin, one of a purulent peritonitis, the others with autopsy findings suggesting those described by Kohler and his colleagues (14) as due to a lack of some substance associated with the "grass juice factor." Seventeen of the group were injected with the same sublethal dose of diphtheria toxin as was used in the first experiment. Five control animals were destroyed at the time the toxin was given to the test group. The suprarenals from four test animals and from a similar number of controls were examined at the end of the 1st and the 2nd days. At this time two of the remaining test animals died. Both had slight gelatinous edema at the site of inoculation of the toxin; one slight, the other marked

TABLE III

Vitamin C Content of Suprarenals of Guinea Pigs Injected with Sublethal Dose of Diphtheria Toxin*

Days after injection of toxin	No. of animals		Vitamin C in test animals		Vitamin C in control animals		Difference between test animals and controls		
	Test	Control	Mean	Standard deviation of mean	Mean	Standard deviation of mean	Difference of means	t	Nearest tabular value P
			mg.	*mg.*	*mg.*	*mg.*	*mg.*		
0		5			0.105	0.021			
1	4	4	0.103	0.008	0.113	0.011	−0.010	0.766	0.5
2	4	4	0.086	0.014	0.122	0.004	−0.036	2.560	<0.05
4†	3	3	0.112	0.003	0.114	0.020	−0.002	0.112	>0.9
7	4	4	0.097	0.026	0.128	0.017	−0.030	0.987	0.4

* The animals were deprived of vitamin C for 7 days, and then given daily subcutaneous injections of 10 mg. of ascorbic acid for another week prior to injection of the toxin.

† Two animals died in slightly more than 2 days. Autopsy findings suggested that death was due to diphtherial intoxication.

congestion of the suprarenals. The lungs of both were congested. The suprarenals of these two animals were not examined for vita-

TABLE IV

Vitamin C Content of Suprarenals of Guinea Pigs Injected with Sublethal Dose of Diphtheria Toxin*

Days after injection of toxin	Vitamin C in test animals		Vitamin C in control animals		Difference between test animals and controls		
	Mean of 5	Standard deviation of mean	Mean of 5	Standard deviation of mean	Difference of means	t	Nearest tabular value P
	mg.	*mg.*	*mg.*	*mg.*	*mg.*		
1	0.088	0.006	0.107	0.012	−0.019	1.466	0.2
2	0.096	0.007	0.103	0.009	−0.007	0.602	0.6
4	0.126	0.009	0.109	0.006	+0.017	1.579	0.2
9	0.113	0.010	0.104	0.013	+0.009	0.549	0.6

* The animals were deprived of vitamin C for 7 days, and then given daily subcutaneous injections of 10 mg. of ascorbic acid for another week prior to injection of the toxin.

TABLE V

Vitamin C Content of Suprarenals of Guinea Pigs Injected with 0.75 of the Uniform Sublethal Dose of Diphtheria Toxin*

Days after injection of toxin	No. of animals		Vitamin C in test animals		Vitamin C in control animals		Difference between test animals and controls		
	Test	Control	Mean	Standard deviation of mean	Mean	Standard deviation of mean	Difference of means	t	Nearest tabular value P
			mg.	*mg.*	*mg.*	*mg.*	*mg.*		
0		5			0.138	0.013			
1	5	5	0.159	0.018	0.160	0.027	−0.001	0.031	>0.9
2	4	4	0.139	0.010	0.148	0.014	−0.009	0.478	0.6
3	5	5	0.112	0.018	0.136	0.015	−0.024	1.005	0.3
7	5	5	0.192	0.022	0.212	0.029	−0.020	0.575	0.6

* The animals received the routine diet except for 5 days beginning 2 weeks before injection of the toxin, when a diet deficient in vitamin C was substituted. Beginning 12 days before and continuing after injection of the toxin, daily doses of 50 mg. of vitamin C were given subcutaneously.

min C. The experiment was continued with the remaining animals. The results are tabulated in Table III. The 29 per cent

decrease in the mean of the vitamin C content of the test animals examined at the end of the 2nd day is significant ($P < 0.05$).

This experiment was repeated in May after the stock animals had been receiving fresh grass for several weeks. Forty animals were prepared and twenty of the group were injected with the sublethal dose of toxin. The results are presented in Table IV. There was no significant difference between the mean vitamin C content of the suprarenals of the test and of the control groups.

In the last experiment, Table V, a definite excess of vitamin C (50 mg. daily) was administered to the animals for 12 days before the injection of 0.75 of the uniform sublethal dose of toxin and thereafter to each animal until it was killed. While this was evident in the greater amount of ascorbic acid in the suprarenals, no significant difference between the mean of this factor in the test and the control groups was recorded.

DISCUSSION

The data presented in this paper are given in terms of the total vitamin C content of the suprarenals, in accordance with the practice of Lyman and King (5) in 1936. Haas (15), who has criticized results expressed similarly from this laboratory (6), presents his figures in terms of ascorbic acid concentration. It would appear that when such a method is used the congestion and edema of the suprarenals of guinea pigs suffering from diphtherial intoxication would materially influence the results.

No significant differences were found between the vitamin C content of the suprarenals of the test animals and the control animals in three of the experiments in which the amount of toxin used was actually a sublethal dose for the level of the susceptibility of the guinea pigs. On the other hand, with the larger doses of toxin a significant difference was observed between the ascorbic acid content of the suprarenals of the test and the control animals, and since both groups received the same amount of the vitamin subcutaneously, this difference would appear to have been a result of the severe intoxication. In the third experiment, two animals succumbed and showed postmortem changes which suggested that death was due to the effects of the toxin after an amount which is not ordinarily fatal had been given. A significant drop in the vitamin C content of the suprarenals of the remaining animals

was recorded. Changes in the ascorbic acid concentration of the suprarenals of the guinea pigs were, therefore, associated only with the administration of lethal doses of diphtheria toxin, an observation that does not suggest the direct combination of diphtheria toxin and vitamin C in the animal body.

The data in Table I as well as those previously published from this laboratory (6) and in Table III of the paper by Haas (15) indicate that under appropriate conditions the total ascorbic acid content of the suprarenals of guinea pigs can increase steadily after the injection of small doses of diphtheria toxin. Such an increase did not result from the intoxication, since an increase also appeared in the controls.

The finding of an increased susceptibility to diphtheria toxin in the guinea pigs used in February, despite the fact that they had been given adequate amounts of vitamin C by subcutaneous injection, suggests that the special diets they received were deficient in some other factor which is related to susceptibility.

SUMMARY

No significant differences were found between the vitamin C content of the suprarenals of guinea pigs injected with a sublethal dose of diphtheria toxin and that of the control animals.

After the injection of a small dose of diphtheria toxin the ascorbic acid content of the suprarenals of a group of guinea pigs and of control animals on the same diet increased steadily for 8 days.

In another experiment a significant decrease in the ascorbic acid content of the suprarenals of a group of guinea pigs occurred 48 hours after the injection of the same dose of diphtheria toxin. This dose while not customarily fatal to animals weighing from 230 to 280 gm. apparently brought about the death of two of the seventeen in the experiment because of a seasonal increase in their susceptibility.

Amounts of diphtheria toxin which are usually fatal were associated with significant changes in the ascorbic acid content of the suprarenals. With those that are always fatal, the changes were even more marked.

It appears, therefore, from these experiments that the smallest dose of diphtheria toxin which will affect the vitamin C content of the suprarenal glands is the least dose which will prove fatal to

some of the test animals and that this dose varies with the susceptibility of the animals.

The problem of demonstrating beyond question that diphtheria toxin and vitamin C do not combine directly in the tissues is an extremely difficult one, but in these experiments no evidence of a direct combination was obtained. The burden of proof must rest with those who hold otherwise, since no evidence of such a combination *in vitro* has been obtained.

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VITAMIN A DEFICIENCY

III. BLOOD ANALYSIS CORRELATED WITH A VISUAL TEST

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Visual tests for measuring vitamin A deficiency assume the existence of a relationship between the rate of regeneration of visual purple and the amount of vitamin A in the blood. The evidence for such a relationship is indirect, since no correlation has yet been reported between any visual adaptation test and the blood level of vitamin A.

The purpose of the investigation reported here was to measure the amount of vitamin A in the blood of subjects whose visual regeneration time had just been measured according to the method of Pett (1). Owing to the uncertainties in analyzing blood for vitamin A (as summarized by Wilbur (2)), a study of available methods was first undertaken. From this study some modifications in procedure were evolved, and a discussion of our methods is necessary before we report the main investigation.

Methods

A comprehensive review and extensive study of this problem and its clinical relationships was reported by Lindqvist (3) in 1938. Valuable comments have since been made by Wilbur (2), Kimble (4), and Thiele and Scharff (5). These methods depend on the well known blue color produced by the reaction of vitamin A (and carotenoids) with antimony trichloride, but the precise manipulations vary widely. Moreover, some workers have stressed the use of a stufenphotometer or a photoelectric colorimeter to get more precise readings of the evanescent blue color.

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Although such instruments may be advantageous, we have found that adequate accuracy can be attained, after sufficient practice, with an ordinary colorimeter.

Our preliminary work led us to adopt Lindqvist's procedure, but, in our hands, certain modifications in the method were found to be necessary. These will be discussed following the description of our procedure.

Procedure

The blood, removed by venipuncture, is centrifuged, and 3 to 5 ml. of plasma (or serum) are measured into each of two 50 ml. Erlenmeyer flasks. One-tenth the plasma volume of 60 per cent alcoholic potassium hydroxide is added, and the flasks are refluxed on a boiling water bath for 5 minutes.

The mixture is quickly cooled to slightly below room temperature and then receives half its volume of 95 per cent ethanol. It is then extracted three times with petroleum ether (b.p. about 50°) to give a total volume of 35 ml. of extract. The petroleum ether extract is washed twice with half its volume of distilled water, dried by shaking with a small amount of anhydrous sodium sulfate, and filtered and washed into a 100 ml. Erlenmeyer flask. The extract is then evaporated at 30–40° with a stream of nitrogen. The residue is taken up in about 1 ml. of petroleum ether, and washed into a small tube (8 × 75 mm.) with a further small portion. This is finally dried with a very small amount (about 0.05 gm.) of sodium sulfate. The extract is filtered into a similar small tube, with a mark at 2 ml., with washing, and brought to 2 ml. volume.

This extract contains the carotene and vitamin A from the original plasma. Since the solution is yellow, due to carotene, and at a definite volume, the amount of carotene can be measured by comparison with a suitable standard. To do this the extract is put in the cup of a Leitz microcolorimeter, and the intensity of yellow color measured against a 10 mm. depth of 0.02 per cent potassium dichromate solution. It is then calculated as β-carotene, though this may not be strictly accurate. The solution is washed back into the graduated tube, with 0.5 ml. of petroleum ether.

Since the antimony reaction takes place in chloroform, the caro-

tene and vitamin A must now be quantitatively transferred to chloroform. The petroleum ether is evaporated off *in vacuo* at room temperature, and the resulting residue taken up in 0.25 ml. of chloroform (U.S.P.). The chloroform solution is transferred to the colorimeter cup and another 0.25 ml. portion used to wash it in. Then 0.50 ml. saturated (30 per cent) antimony trichloride in chloroform is added and the resulting blue color matched against a 10 mm. depth of 5 per cent copper sulfate. This blue

TABLE I

Effects of Temperature and Time on Liberation of Bound Vitamin A in Human Plasma

Temperature of refluxing water bath	Time of refluxing	Vitamin A
°C.	min.	I.U. per 100 ml.
Boiling (97°)	30	18.4
"	30	19.9
"	30	19.4
"	15	43.5
"	15	43.1
"	15	43.1
"	5	46.4
"	5	46.2
"	5	45.4
50	30	28.0
50	30	27.6
50	30	27.8
50	60	41.1
50	60	43.3
50	60	42.7

color is due to carotene as well as vitamin A, and must be corrected for the amount of carotene found above.

Influence of Heat—Lindqvist (3) showed the importance of a preliminary saponification of the plasma, apparently to remove vitamin A from some complex.[1] However, the minimum time he

[1] The nature of the complex binding vitamin A is of some interest, and preliminary work with fractional precipitation suggests that it may be serum globulin. An alcoholic precipitate of one blood sample showed 400 I.U. of vitamin A (spectrographically determined) in the precipitate and only 250 in the alcohol.

investigated, 30 minutes, destroys vitamin A, as shown in Table I; the table also shows that suitable results can be obtained by longer heating at lower temperatures. It will also be noted that the consistency of results is better with shorter heating times.

Extraction with Petroleum Ether—Lindqvist extracts the saponified plasma with diethyl ether and after several manipulations transfers the extracted material to petroleum ether for measuring the yellow color. Equally good results and quite a saving in time were obtained by direct extraction with petroleum ether, as shown in Table II.

Calculations—The calculations involve (1) a comparison of the yellow dichromate standard with pure carotene in solution; (2) a comparison of the blue copper sulfate standard with the blue

TABLE II

Comparison of Extraction Process in Lindqvist and Present Methods

Serum No.	Lindqvist method, ethyl ether + petroleum ether		Present method, petroleum ether only	
	β-Carotene	Vitamin A	β-Carotene	Vitamin A
	γ per 100 ml.	*i.u. per 100 ml.*	*γ per 100 ml.*	*i.u. per 100 ml.*
1	2.6	41.1	2.5	39.0
	2.7	40.0	2.9	39.4
2	2.9	36.6	2.8	35.5
	3.1	37.2	3.0	36.5

color developed by pure vitamin A; and (3) ascertaining the amount of blue color developed under the conditions by a given amount of carotene.

Pure β-carotene (kindly supplied by the S.M.A. Corporation) was weighed and dissolved in petroleum ether to give a standard solution. It was found that a solution containing 1.12 γ per ml. at a depth of 10 mm. gave a yellow color equal to a 10 mm. depth of 0.02 per cent dichromate.

A vitamin A solution in chloroform, free of carotene, was prepared from a pure sample, the potency of which had been determined spectrographically by the makers and by us. This solution was used to develop a blue color for comparison with 5 per cent copper sulfate solution. It was found that 1.83 I.U. of vitamin

A per ml. give a blue color equivalent to 10 mm. of 5 per cent copper sulfate when matched at 10 mm. depth.

It was also found that 88.0 γ of β-carotene give the same amount of blue color as 84.1 I.U. of vitamin A, under our conditions.

The validity of a colorimetric method must be confirmed in several ways.

1. The intensity of color at different depths or concentrations should follow Beer's law by showing a proportional change as the concentration changes. That the estimation of yellow color in the present method follows this requirement is shown by the following results obtained when β-carotene solutions were matched against 0.02 per cent potassium dichromate solution, 10 mm. in depth.

Concentration of carotene solution, γ *per ml.*	4.48	2.24	1.12
Depth of carotene solution, *mm*...	2.8	5.5	10.6

Similar results were obtained in the development of a blue color when varying known amounts of vitamin A were matched against 5 per cent copper sulfate solution, 10 mm. in depth.

Vitamin A, *i.u. per ml.* . .	1.83	2.44	3.05	4.55	6.10
Colorimeter reading, *mm.*	10.0	6.7	5.9	4.1	3.1

2. The method must continue to show this proportionality in the presence of plasma; *i.e.*, under actual working conditions. This was tested by adding known amounts of carotene solution or of vitamin A solution to plasma (or serum) and following the procedure given above. The range covered and the values obtained were substantially the same as shown in the tabulations above, so no additional information would be provided by extra tables. Further verification of compliance with Beer's law, in the presence of plasma, is given in the following figures.

Plasma used, *ml.*	10.0	7.5	5.0
Vitamin A found, *i.u.*.............	4.17	3.10	2.06

3. The method in this case is not finally confirmed until it has been shown that changes in the blood level of vitamin A following administration or deprivation of vitamin A in a human being are detected by it. This proof is reserved for the next section, since it is also concerned with the main object of this study; namely, the interdependence of the diet, the blood level of vitamin A, and the Pett adaptation test.

EXPERIMENTAL

In all cases the subject was given the test described by Pett (1), and a sample of 10 to 20 ml. of blood was taken from an arm vein into a syringe containing solid sodium oxalate. The method already given was then followed in analyzing the blood.

	0 hr.	4 hrs.
Vitamin A, *i.u. per 100 ml.*	76	133
Recovery time, *sec.*	9	5.7

It is seen that 4 hours after the ingestion of 20,000 I.U. of vitamin A our method reveals a significant rise in the level of the vitamin in the blood. The rise indicates that about 10 per cent of the administered vitamin is in the circulation but we have not as yet sufficient observations on absorption or removal to discuss this point. In any case the figures above further indicate that the visual recovery time is significantly reduced, as expected, from the increased vitamin A level of the blood.

Table III shows that both a fall and a rise in the blood vitamin A can be detected by this method, when they are varied as a result of the diet; Table III also shows the close relation of the blood level with the visual test.

While this latter experiment was going on, various people, with different adaptation times, were bled as mentioned above, and the blood vitamin A determined. A total of twenty-six persons was so studied. The blue values obtained were corrected for the carotene found in the plasma, but the correction was never more than about 2 per cent of the vitamin A value. These subjects were all males, of ages from 20 to 30 years, in good health, not under treatment nor on special diets. Thus it was hoped that the chief variable would be the "recovery times" in the visual test.

TABLE III

Relation of Blood Vitamin A and Vitometer Value (Recovery Time in Seconds) for an Individual on Vitamin A-Free Diet

Days from start	Recovery time	Vitamin A per 100 ml. plasma
	sec.	*i.u.*
0	6	87.8
2	8	
4	9	
6	10	
8	13	
10	15	
11	16	
12. 10.10 a.m.	17	66.4
10.15 " 8500 I. U. vitamin A orally		
10.55 "	14	
11.10 "	10	
11.25 "	9.8	117.0
12.10 p.m. (2 hrs.)	9	
1.35 "	12	
4.35 "	11	
5.35 "	13	
7.35 " (9 hrs.)	14	64.1
13. 9.50 a.m.	15	57.5

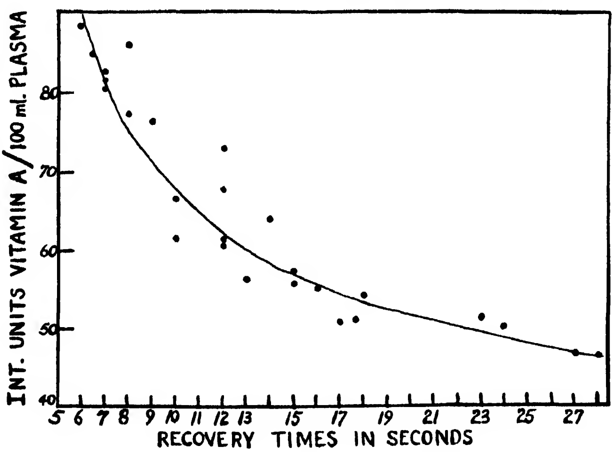

FIG. 1. Curve showing the relationship in the blood content of vitamin A by direct determination and by the Pett test for vitamin A deficiency.

The results, given in Fig. 1, show a good correlation between the adaptation test and the blood level of vitamin A. The curve is a rectangular hyperbola, with the equation $y = (311/x) + 36.1$, where y is plasma vitamin A.

DISCUSSION

The points about the curve in Fig. 1 show considerable dispersion. While this might be due to inherent errors in the adaptation test or the blood analysis or both, there is a possible physiological explanation worthy of consideration. Our observations suggest that all the widely divergent points represent persons not in an equilibrium condition. This means that the blood level of vitamin A was changing, either up or down, owing to absorption or to transference of vitamin A into the tissues, and the adaptation test is not sensitive enough to detect this fact. In this connection it may be pointed out that we have some evidence of a diurnal rhythm for the level of vitamin A in the blood.

Since it was not possible to control all these factors in the twenty-six persons studied, the fact that most of the points follow the curve closely is all the more significant. It means that small variations of normal physiological type, detectable by blood analysis, do not register sufficiently on the visual test to bias interpretation. As pointed out by Pett (1), this adaptation test has been limited to a certain degree of sensitivity so as to be very rapid and simple in operation, and thus of greatest use in routine clinical practice.

The form of this curve is of some physiological interest. While it is easier to think of correlations in terms of a straight line, this is not possible here, since it would cut the axes, thereby representing zero or even negative amounts of vitamin A and adaptation times. This curve suggests that a person may exist at a certain low blood level of vitamin A, with a retarded recovery time as a kind of subsistence level. To build such a person up to what may be the optimal level requires a large amount of vitamin A and nearly double the blood level. Such have, indeed, been the findings on treatment already reported. Considerable interest attaches to this question of the dividing line between optimal and suboptimal blood levels. As suggested by Pett (1), a survey of any group of people includes types falling on two different dis-

tribution curves, and a given value may be normal for one person, and deficient for another. Nevertheless, evidence was presented showing a fairly clear separation into two groups, on somewhat arbitrary bases. The form of the curve in Fig. 1 bears out this general idea of two groups of persons—one at each asymptote—and a transition zone. The ultimate criterion of deficiency must still be response to administration of vitamin A, and the visual test used here seems to be sensitive enough and much more rapid than any other for following the effects of administration.

SUMMARY

1. A definite correlation has been found between the vitamin A level in the blood, as determined by a somewhat new method, and the Pett visual test for vitamin A deficiency.

The authors express sincere gratitude to Dr. M. M. Cantor for assistance in obtaining the blood samples; to Miss Marian K. Lipkind for performing the adaptation tests; and to the subjects of the experiments.

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THE COLORIMETRIC ASSAY OF TOTAL, α-, AND β-17-KETOSTEROIDS IN EXTRACTS OF HUMAN URINE*

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The present paper reports a method for determining the respective concentrations of total, α-, and β-17-ketosteroids in urine which is based upon the previously reported method of assaying mixtures of crystalline androsterone (α-steroid) and dehydroisoandrosterone (β-steroid) (1) and a simple means of eliminating the interfering effect of the non-ketonic substances from the colorimetric determination.

The commonly employed procedure for "clearing" urine extracts with decolorizing charcoal (2) may result in the loss of significant amounts of hormones (3). On the other hand, urine contains non-ketonic substances which have a color with an absorption spectrum in the same region as that developed by 17-ketosteroids with *m*-dinitrobenzene and potassium hydroxide in alcoholic solution. Therefore, either these non-ketonic substances must be eliminated or the light absorption due to them must be subtracted in the colorimetric assay. The method reported here adopts the latter procedure. In presenting the evidence for the validity of this correction, a procedure is also described for eliminating the non-ketonic substances.

The application of the digitonin precipitation procedure to the detailed quantitative analysis of 17-ketosteroids in the neutral fraction of urine extracts is based upon the fact that steroids which have a hydroxyl group in the β position (4) on carbon atom 3 are selectively precipitated with this reagent. The latest evidence to be found in the literature (5, 6) suggests that only two β-17-ketosteroids exist in significant amounts in the neutral fraction

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of urine extracts. The first, dehydroisoandrosterone, is present in small amounts in normal urines (5, 7). The second, isoandrosterone, has been isolated only from the urine of patients with adrenal disease (6). Although it is not justifiable to state that the β-17-ketosteroid fraction is composed entirely of dehydroisoandrosterone, it is reasonable to conclude that the determination of β-steroids gives the upper limit of concentration of that hormone.

EXPERIMENTAL

Preparation of Extracts for Colorimetric Assay—The collection, hydrolysis, and extraction of urine were carried out according to the procedure described previously (3).[1] After the benzene was evaporated off, the extract was dissolved in 100 cc. of ethyl ether. The acidic and phenolic fractions were removed by washing the ether solution four times with 20 cc. portions of 10 per cent aqueous NaOH and three times with 20 cc. portions of water. An aliquot of the remaining ether solution was treated in the following three ways. (*a*) One portion was evaporated to dryness and the residue dissolved in a measured amount of absolute alcohol. This alcoholic solution (dark red) is hereafter referred to as the *crude fraction.* (*b*) To another portion of the ether solution, decolorizing charcoal (norit A, Pfanstiehl) in the proportion of 0.5 gm. per 100 cc. of ether solution was added. The mixture was stirred mechanically for 3 minutes. The decolorized ether solution was filtered from the charcoal through Whatman No. 50 filter paper with the aid of suction. The charcoal was washed once with 20 cc. of fresh ether and the combined ether extracts were evaporated to dryness. The residue (essentially colorless) dissolved in a measured amount of absolute alcohol is hereafter referred to as the *charcoal-treated fraction.* (*c*) In a final aliquot, the ether solution was evaporated to dryness and the residue was divided into ketonic and non-ketonic fractions by the following modification of the Butler and Marrian procedure (6). The dry residue was dissolved in 4 cc. of 95 per cent ethyl alcohol, and after addition of 0.5 cc. of glacial acetic acid and 0.5 gm. of Girard's Reagent T,[2]

[1] We are indebted to Dr. I. Nathanson for samples of urine extract from the girl with adrenal tumor.

[2] We are indebted to Dr. J. Wolfe for a generous supply of this reagent and for valuable advice in connection with this procedure.

the solution was refluxed for 1 hour on the water bath. After the solution had been cooled and 40 cc. of ice water added, 3 cc. of 10 per cent aqueous NaOH were added and the mixture was extracted four times with 40 cc. portions of ethyl ether. The combined ethereal extracts, after being washed three times with 20 cc. portions of water, were evaporated to dryness. The residue was dissolved in a measured amount of absolute alcohol. This alcoholic solution (dark red) is hereafter referred to as the *non-ketonic fraction.*

1 cc. of concentrated sulfuric acid diluted with the water washings of the above ether extract plus 20 cc. of ethyl ether was added to the aqueous phase remaining after the ether extraction of the non-ketonic fraction. After standing for at least 2 hours at room temperature, the mixture was extracted four times with 40 cc. portions of ether. Later it was found that the recovery of ketones was more complete if 1 additional cc. of sulfuric acid was added just prior to the ether extraction. The ethereal extract, after being washed three times with 20 cc. portions of water, was evaporated to dryness. The residue was dissolved in a measured volume of absolute alcohol. This solution (essentially colorless) is hereafter referred to as the *ketonic fraction.*

Determination of β-17-Ketosteroids—The procedure previously described for dehydroisoandrosterone in the presence of androsterone (1) was used with the single change that the residue from the benzene extract of the supernatant was treated twice with ether instead of once in order to insure that all the acetone had been evaporated off.

Colorimetric Assay—The modified Callow procedure previously described (8) was adapted for the Evelyn photoelectric colorimeter without other change. A measured quantity of a solution of urine extract was placed in a colorimeter tube and the volume brought up to 0.20 cc. by addition of absolute alcohol. 0.20 cc. of a solution of *m*-dinitrobenzene (11.6 mg. per cc.), and then 0.20 cc. of a 2.5 N KOH, both in absolute alcohol, were added. The mixture and a blank solution prepared in the same way, but containing no hormone, were placed in a constant temperature bath at 25.0° ± 0.2°, and allowed to develop for 80 minutes. On removal from the bath the test and blank reaction mixtures were diluted by the addition of 20.0 cc. of 95 per cent ethyl alcohol. The galvanometer reading of the colorimeter, with a Rubicon

No. 520 filter with transmission limits of 470 to 580 and a maximum at 525 mμ was adjusted to 100 with the blank solution. On removal of this tube the galvanometer reading after each blank setting should fall to the same reading within 3 units. The galvanometer reading (G_1) of the test solution was then obtained. The 17-ketosteroid content of the test solutions was estimated by referring to a calibration curve in which galvanometer readings

TABLE I

Sample Calibration Curve. Data Obtained with Crystalline Androsterone and Dehydroisoandrosterone

Sample	Concentration per cc.	Galvanometer reading	K*
	γ		
Androsterone	14.56	12.0	0.063
	9.71	25.0	0.062
	6.50	37.0	0.066
	3.25	60.0	0.068
	1.94	73.0	0.070
	0.97	86.5	0.065
			0.066 (Average)
Dehydroisoandrosterone	14.45	12.5	0.064
	9.64	23.0	0.066
	6.45	36.5	0.066
	3.22	60.0	0.069
	1.93	74.0	0.068
	0.96	86.5	0.066
			0.067 (Average)
Androsterone and dehydroisoandrosterone	6.48	37.0	0.067
	1.94	73.5	0.069
			0.068 (Average)

* The amounts of androsterone and dehydroisoandrosterone in the original solutions from which these dilutions were made were weighed with an accuracy of 0.1 per cent.

were plotted against known amounts of crystalline androsterone and dehydroisoandrosterone (0.05 to 0.3 mg.). When an appreciable amount of crude colored substance was present in the alcoholic solution tested, a correction for this crude color was made as follows: A quantity of the solution equal to that used for the 17-ketosteroid determination was added to the developed and diluted blank and the galvanometer reading (G_2) was *im-*

mediately obtained. The mg. of sterone corresponding to this reading on the calibration curve subtracted from the value obtained from galvanometer reading G_1 gave the corrected mg. of sterone. Or, since the concentration of 17-ketosteroids in the solution in the colorimeter is described by $C = K(2 - \log G)$, the corrected concentration may be calculated as $C_c = K(\log G_2 - \log G_1)$.

By the method described above androsterone (m.p. 181.5–182.7°),[3] dehydroisoandrosterone (m.p. 149–150°), and combinations of the two in concentrations ranging from 1 to 15 γ per cc. in the colorimetric solution give a K value of 0.067 ± 0.004 (Table I). These data, taken from a sample experiment in the course of establishing our calibration curve, show that the expected error in the colorimetric determination is approximately 6 per cent. The constancy of the K value for androsterone, dehydroisoandrosterone, and combinations of the two is in contradiction to the findings of Friedgood (9). We assume that this difference is due to differences in the colorimetric procedures employed.

Hormones—Samples of crystalline androsterone and dehydroisoandrosterone were obtained through the kindness of Dr. Erwin Schwenk of the Schering Corporation. They were used without further purification.

Results

The data of Table II present determinations of total and α-17-ketosteroids in alcoholic urine extracts before and after known amounts of crystalline androsterone and dehydroisoandrosterone had been added. The β-steroid values were calculated by difference between the total and α-17-ketosteroids. The values obtained on the charcoal-treated, crude, ketonic, and non-ketonic fractions before hormone was added are given first. The theoretical values represent the sum of the determined values and the mg. of hormone added. The corresponding determined values are listed under the first determined value. It will be seen that the theoretical and determined values agree satisfactorily. This indicates that α- and β-steroids may be assayed as accurately in alcoholic urine extracts as in pure alcohol solutions.

[3] We are grateful to Dr. J. Wolfe for the melting point determinations.

The data in Table III show the distribution of a measured amount of crystalline 17-ketosteroid after treatment with Girard's Reagent T between the ketonic and non-ketonic fractions. It is evident that the non-ketonic fractions are essentially free from steroids, while the ketonic fractions contain about 90 per cent

TABLE II

Recovery of Known Amounts of Crystalline Androsterone and Dehydroisoandrosterone Added to Crude, Charcoal-Treated, Ketonic, and Non-Ketonic Fractions of Normal Male Urine

The results are expressed as mg. per cc. of alcoholic solution.

Urine fraction	Sterone added	Total 17-ketosteroids		α-17-Ketosteroids		β-17-Ketosteroids*	
		Theoretical	Determined	Theoretical	Determined	Theoretical	Determined
1. Charcoal-treated	None		1.3		1.3		0.0
	1.2 A.	2.5	2.4	2.5	2.4	0.0	0.0
	1.5 D.A.	2.8	2.6	1.3	1.1	1.5	1.5
2. Crude	None		1.4		1.3		0.1
	0.8 D.A.	2.2	2.2				
	1.5 "	2.9	2.7				
	0.2 "	1.6		1.3	1.3	0.3	0.3
	0.2 "	1.6		1.3	1.3	0.3	0.3
2. Ketonic	None		0.9		0.9		0.0
	1.0 D.A.	1.9	1.9	0.9	0.9	1.0	1.0
2. Non-ketonic	None		0.2				
	1.1 D.A.	1.3	1.3				
	0.6 "	0.8	0.8				
3. Ketonic	None		2.0		0.9		1.1
	0.4 A.	2.4	2.3	1.3	1.2	1.1	1.1
3. Crude	None		1.5		0.7		0.8
	0.7 A.	2.2	2.1	1.4	1.3	0.8	0.8

A. = androsterone; D.A. = dehydroisoandrosterone.

* Calculated by the difference between total 17-ketosteroids and α-17-ketosteroids.

of the theoretical yield. This evidence indicates that Girard's Reagent T effectively separates ketonic substances.

The comparison of the mg. per unit volume of urine of total, α-, and β-17-ketosteroids obtained from analysis of the crude, charcoal-treated, ketonic, and non-ketonic fractions of urine extracts is presented in Table IV. The crude fraction consist-

TABLE III

Recovery of Known Amounts of Crystalline Androsterone and Dehydroisoandrosterone in Ketonic Fraction Obtained after Treatment with Girard's Reagent T Expressed As Total Mg. of Hormone

Experiment No.	Hormone	Ketonic fraction: Theoretical (1)	Ketonic fraction: Determined (2)	Ketonic fraction: Ratio (2):(1)	Non-ketonic fraction determined (theoretical, 0)
5	Dehydroisoandrosterone	4.0	3.6	0.90	0.5
6	Androsterone	4.0	3.4	0.85	0.1
7	Dehydroisoandrosterone	2.8	2.4	0.86	0.1
8	"*	6.3	6.9	1.09	0.2
9	Androsterone*	6.3	6.0	0.95	0.3

* In these experiments an additional cc. of sulfuric acid was added before extraction of the ketonic fraction, as described in the text.

TABLE IV

Comparison of Mg. per Unit Volume of Urine of α- and β-17-Ketosteroids As Determined on Crude, Charcoal-Treated, Ketonic, and Non-Ketonic Fractions of Urine Extracts*

Type of urine	Fraction	Totol sterone	α-Steroids	β-Steroids†
Normal adult male, Sample A	Crude	16.2	15.0	1.2
	Ketonic	13.9	13.9	0.0
	Non-ketonic	0.2		
	Charcoal-treated	9.0	7.6	1.4
Normal adult male, Sample B	Crude	16.0	13.6	2.4
	Ketonic‡	16.0	13.1	2.9
	Non-ketonic	0.6		
	Charcoal-treated	10.5	9.5	1.0
Girl with carcinoma of adrenal cortex	Crude	167	63	104
	Ketonic	151	54	97
	Non-ketonic	3.5		
	Charcoal-treated			
	0.25 gm.	130	51	79
	1.00 "	88	38	50

* Approximately 1 liter for the normal adult male urine and the 24 hour amount for the girl patient.

† Calculated by difference.

‡ In these experiments an additional cc. of sulfuric acid was added before extraction of the ketonic fraction, as described in the text.

ently gives the highest values. The values obtained from the ketonic fraction represent about 90 per cent of the values obtained on the crude fraction, as would be expected. The sterone content of the non-ketonic fraction is very low. The charcoal-treated fractions give values for the male urines about 33 per cent lower than the crude and for the urine from the girl 20 per cent lower when 0.25 gm. of charcoal was used and 44 per cent lower when 1.0 gm. of charcoal was used. Thus there is a significant loss of hormone coincident to treatment with this decolorizing charcoal, which apparently varies in proportion to the amount of charcoal used.

Comments

The data obtained lead to the following conclusions.

Total, α-, and β-17-ketosteroids may be determined with reasonable accuracy on crude alcoholic solutions of the neutral fraction of urine extracts with the procedure described here.

Purification of neutral extracts with Girard's reagent may be carried out without loss of a significant amount of 17-ketosteroids.

Clearing extracts with decolorizing charcoal is unnecessary and may entail the loss of significant amounts of hormone.

The total, α-, and β-17-ketosteroid content of urine extracts may be determined with reasonable accuracy on either the crude or ketonic fractions.

The accuracy of these determinations in measuring the hormone content of original urine will depend upon establishing the optimal conditions for hydrolysis and extraction.

SUMMARY

A colorimetric method of assaying total, α-, and β-17-ketosteroids is described. Two methods for eliminating interfering substances and the error which may be caused by treatment with decolorizing charcoal are presented.

We are indebted to Dr. L. Fieser and Dr. J. Wolfe for valuable suggestions during the course of this investigation.

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ESTROGENS WITH OXYGEN IN RING B

II. Δ_6-ISOEQUILIN FROM 7-HYDROXYESTRONE*

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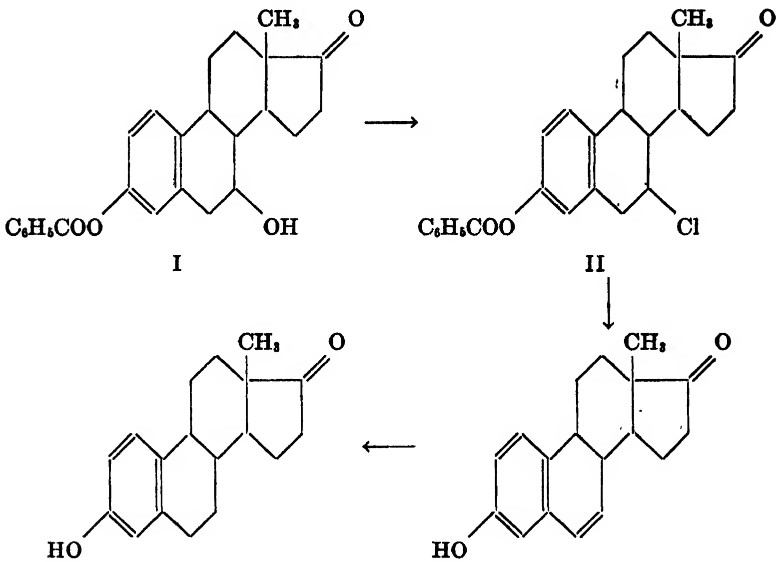
The original object of the work described in this series of papers was the synthesis of Δ_{6-7} double bond isomers of equilin or 17-dihydroequilin, which we needed for spectroscopic comparison with a diol (1) isolated from the urine of pregnant mares. This necessitated the preparation of estrogens substituted with oxygen in positions 6 or 7 as intermediates. The transformation of equilin into 7-hydroxyestrone has been described in Paper I (2). The present report deals with the conversion of the latter compound into one of the desired products, Δ_6-isoequilin.

An attempt to dehydrate 7-hydroxyestrone directly to the Δ_6 isomer of equilin, by heating in the presence of aluminum oxide, was unsuccessful. The customary method of establishing the double bond by splitting out benzoic acid from the 7-benzoate was not tried because it likewise necessitates high temperatures, which usually are detrimental to the yield. We therefore decided on an alternative pathway; namely, replacement of the 7-hydroxyl group by chlorine and subsequent elimination of hydrochloric acid. 7-Chloroestrone benzoate (II) was obtained in excellent yield from 7-hydroxyestrone-3-monobenzoate (I) by the method of Westphal, Wang, and Hellmann (3), which employs phosphorus pentachloride in chloroform solution with calcium carbonate as acid-binding agent. Treatment with thionyl chloride in pyridine, which was also tried, proved less satisfactory in regard to yield and purity of the product. The elimination of

* This report is from a dissertation submitted by W. H. Pearlman in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

hydrochloric acid from the chloro derivative was effected by heating in pyridine solution containing sodium iodide. After removal of the benzoyl group by alkaline hydrolysis a compound melting at 266°[1] and possessing the analytical composition of equilin was obtained in good yield (III). The specific rotation of the new equilin isomer is +150°, which is less than half the value given by equilin in the same solvent (+308°). The presence of a double bond in the α,β position to Ring A follows from the absorption spectrum. The absorption curve (Fig. 1, Curve 1) exhibits two



I II

IV III

distinct maxima at 263 (ϵ = 7500) and 306 mμ (ϵ = 2500). A double inflection, indicative of a secondary maximum, is visible at about 272 mμ. In its general characteristics it thus resembles the spectrum of 6-ketoestradiol,[2] although the positions of the maxima are not the same.

Δ_6-Isoequilin yields a benzoate melting at 202° and an acetate melting at 141°. The new equilin isomer was found to possess about one-third of the physiological potency of estrone.[3]

[1] All melting points reported in this paper are corrected.
[2] Wintersteiner, O., unpublished data.
[3] The assays were carried out in the Biological Laboratory of the Scher-

When equilin is treated with hydrogen activated by palladium black, dehydrogenation occurs simultaneously with hydrogenation (4). The reaction products are equilenin (5) and a stereoisomer of estrone in which, as analogous experiments by Serini and Logemann (6) on 17-dihydroequilin indicate, carbon atom 8 is sterically inverted (8-epiestrone, isoestrone). Our new equilin isomer, on the other hand, yields on hydrogenation with palladium estrone (IV) as the sole product. We convinced ourselves of the absence of equilenin by spectroscopic examination of the crude hydrogenated product; the absorption curve is of the simple phenolic type and exhibits none of the bands characteristic for the naphtholic estrogen. The behavior of the new isomer on hydrogenation is fully consistent with the position assigned to the double bond.

The conversion of 7-hydroxyestrone into estrone by the chain of reactions described above conclusively establishes the configuration of carbon atom 8 in the 7-substituted estrogens as that present in the natural steroids. Our previous evidence bearing on this point was based on a reaction in which a stereochemical inversion at C_8 may conceivably have taken place; namely, on the Wolff-Kishner reduction of 7-ketoestrone to 17-desoxoestrone (2). Since in the reactions described here as well as in the foregoing reduction of 7-ketoestrone to 7-hydroxyestrone carbon atom 8 is in no way involved, this objection does not apply here.

The properties of Δ_6-isoequilin would seem to preclude its identity with the isoequilin which Inhoffen (7) obtained from 5,6(?)-dibromoandrostanedione-3,17 by debromination and subsequent heat treatment. Neither do our data fit the description given by Girard and coworkers (8) of hippulin, a supposed isomer of equilin which was isolated by these authors from the urine of pregnant mares. The absorption spectrum of hippulin has not been measured, while Inhoffen reports for his isomer only the location of the absorption bands (265, 275, and 334 mμ) but not their intensity. The fact that Δ_6-isoequilin absorbs around 263 mμ, isoequilin A (Fig. 1, Curve 3) (9) around 273 mμ, may indicate that Inhoffen's substance, which he believes to be either a Δ_6- or a Δ_{8-9}-equilin, is possibly a mixture containing

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isomers of both these types. On the other hand, neither Δ_6-isoequilin nor isoequilin A shows any absorption around 334 mμ. There are only two other types of estrogens whose spectra exhibit bands in the region from 320 to 350 mμ; namely, 6 keto-estrone and 6-ketoestradiol, and equilenin and the dihydro-equilenins. All that can be said at present is that the available spectrographic evidence is not wholly consistent with either of the alternative structural formulæ considered by Inhoffen.

There remains to be discussed the possible structural relation-

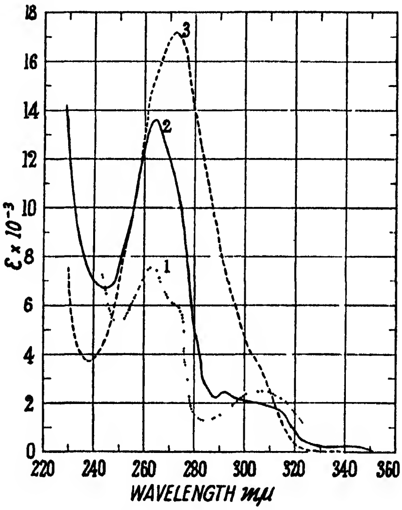

FIG. 1. Absorption spectra of Δ_6-isoequilin (Curve 1), Compound 3 from the urine of pregnant mares (Curve 2), and isoequilin A (Curve 3). Solvent, ethyl alcohol.

ship of Δ_6-isoequilin to the diol isolated from the urine of pregnant mares by Hirschmann and Wintersteiner (1), designated in their paper as Compound 3. At present this question also can be examined only on the basis of the spectrographic findings. It need hardly be mentioned that the nature of the functional group at C_{17}—ketonic or alcoholic—is immaterial in this respect. The spectrum of the diol (Fig. 1, Curve 2) clearly indicates the presence of a double bond in α,β position to the phenolic ring. The maximum of the main band is located at nearly the same wavelength as in the spectrum of Δ_6-isoequilin; namely, at 264 mμ.

However, ϵ (maximum) of this band is nearly twice as high for the urinary diol as for the new Δ_6 ketone. In the region from 285 to 310 mμ the curve of the diol flattens out without revealing any distinct bands, while that of Δ_6-equilin shows a well defined maximum at 306 mμ. Although about 8 per cent of β-dihydroequilenin was present in the preparation of the diol, the contribution to light absorption from that source is negligible in this region. A general similarity of the two curves in regard to the locations where light is absorbed cannot be denied, but the quantitative differences are too pronounced to lend support to the view that the diol is a Δ_6 compound. We are now forced to include in our considerations the only remaining possibility for the location of the double bond in the diol, namely position 9–11, which is likewise α,β to Ring A. Indeed, the spectrum of the diol bears a fair resemblance to that of a compound described in a previous publication (9), to which we assigned the structure of a 14-epi-Δ_{9-11}-8-hydroxyequilin. In any case it will be necessary to isolate larger amounts of the difficultly accessible diol and to supplement the spectrographic evidence, which is inconclusive at present, by chemical studies, especially hydrogenation and dehydrogenation experiments.

EXPERIMENTAL

7-Chloroestrone-3-Monobenzoate—29.3 mg. of 7-hydroxyestrone-3-monobenzoate, m.p. 180–181°, were dissolved in 2 cc. of dry chloroform and 30 mg. of dry, pulverized calcium carbonate added. The mixture was chilled to 0° and 30 to 35 mg. of freshly sublimed phosphorus pentachloride added in small portions with shaking during the course of 20 minutes. After about 10 minutes the reagent was decomposed with 2 cc. of cold saturated sodium bicarbonate solution, and the reaction product extracted with chloroform. The chloroform extract was dried with sodium sulfate, filtered, and evaporated. The white crystalline residue from the dried chloroform solution was recrystallized twice from absolute alcohol with a few drops of benzene. 19.5 mg. melting at 247–248° with decomposition were obtained.

Repetition of the experiment with larger amounts gave a slightly higher yield (70 per cent) of pure material.

Analysis—$C_{25}H_{25}O_3Cl$.	Calculated.	C 73.43, H 6.16, Cl 8.67
	Found.	" 73.24, " 6.24, " 8.28, 8.36

Δ_6-*Isoequilin*—82 mg. of 7-chloroestrone benzoate and 300 mg. of sodium iodide were dissolved in 1.5 cc. of dry pyridine. The solution was heated in a sealed tube on a steam bath for 40 hours. On addition of 8 cc. of water a crystalline precipitate separated which was centrifuged and washed repeatedly with water. The crude product melted at 187–192°. A preliminary experiment had shown that this product still contained small amounts of the starting material. Since the latter is much less soluble in alcohol than Δ_6-isoequilin benzoate, recrystallization at this stage serves no purpose. The crude benzoate was therefore hydrolyzed by boiling for 40 minutes with 5 per cent methyl alcoholic potassium hydroxide. The slightly yellow solution was made weakly acidic with hydrochloric acid and, after dilution with water, extracted with ether. The ether extracts, after being washed with bicarbonate solution and water, were brought to dryness. The crystalline residue (43 mg.) was recrystallized three times from absolute alcohol, yielding 30 mg. of elongated plates with oblique ends, m.p. 265–266°. $[\alpha]_D^{24} = +150°$ (0.93 per cent in dioxane).

Analysis—$C_{18}H_{20}O_2$. Calculated, C 80.55, H 7.52; found, C 80.46, H 7.46

Δ_6-*Isoequilin Acetate*—23 mg. of Δ_6-isoequilin were dissolved in 2 cc. of dry pyridine and 1 cc. of acetic anhydride. The solution was permitted to stand overnight at room temperature. On addition of water the ester separated in crystalline form. This precipitate was centrifuged and washed repeatedly with water (24 mg., m.p. 138–139°). Recrystallization from 90 per cent alcohol yielded 19 mg. of needles melting at 140°. The analysis of this product was 1 per cent too low in carbon. The acetate was therefore hydrolyzed with alkali to the free compound (m.p. 266°), which on analysis gave satisfactory figures (C 80.30, H 7.52), as did the original sample. The acetylation experiment was then repeated under the same conditions, but for the final recrystallization a mixture of benzene and pentane (1:10) was used instead of dilute alcohol. This preparation (m.p. 140–141°) analyzed correctly after being dried at 110° and 8 mm.

Analysis—$C_{20}H_{22}O_3$. Calculated, C 77.38, H 7.15; found, C 77.16, H 6.90

Probably some alcohol was retained in the first preparation in spite of the rigorous conditions of drying.

Δ_6-Isoequilin Benzoate—13 mg. of Δ_6-isoequilin were dissolved with warming in 10 cc. of 1 N sodium hydroxide. The colorless solution was permitted to cool and 0.25 cc. of benzoyl chloride was then added in three portions with vigorous shaking. The precipitate was washed well with water. On crystallizing once from absolute alcohol 14 mg. of long white needles were obtained, which melted at 200–201°. Two more recrystallizations yielded 9 mg. which melted sharply at 202°. For analysis the preparation was dried for 8 hours at 110° and 0.01 mm. to constant weight.

Analysis—$C_{25}H_{24}O_3$.	Calculated.	C 80.63, H 6.50
	Found.	" 79.87, 80.04, H 6.45, 6.40

The deficit in the carbon figure is probably due to retention of solvent, since the sample of Δ_6-isoequilin from which the benzoate was prepared gave the correct analysis.

Estrone from Δ_6-Isoequilin—7.7 mg. of Δ_6-isoequilin recovered from the mother liquors (m.p. 261–263°) were dissolved in absolute alcohol and hydrogenated in the presence of palladium black. On evaporation of the filtered solution 7.3 mg. of a crystalline residue were obtained, which on recrystallizing once from alcohol yielded 6.2 mg. melting at 256–257°. The determination of the specific rotation was carried out in a 1 dm. micropolarization tube. $[\alpha]_D^{25} = +150°$ (0.92 per cent in dioxane).

The ultraviolet absorption spectrum was identical with that of estrone (maximum at 283 mμ, $\epsilon = 2200$).

The material was recovered and recrystallized to yield 4.8 mg. melting at 256–257°. A melting point of 258–259° was observed when the product was mixed with an authentic sample of estrone, m.p. 259°.

Analysis—$C_{18}H_{22}O_2$. Calculated, C 79.97, H 8.20; found, C 79.61, H 8.20

SUMMARY

The preparation of Δ_6-isoequilin from 7-hydroxyestrone is described. The new equilin isomer is not identical with the isoequilin of Inhoffen. Unlike equilin it yields estrone on catalytic reduction. Δ_6-Isoequilin possesses about one-third of the physiological potency of estrone.

The microanalyses reported in this paper were carried out by Mr. William Saschek.

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RELATIVE AMOUNT OF HEPATIC GLYCOGEN DEPOSITED BY GLUCOSE, GLYCINE, AND *dl*-ALANINE

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It was shown long ago that glycine and *dl*-alanine are both converted entirely to glucose in the phlorhizinized dog (1). If these compounds are converted to glucose as a step in their metabolism by the *normal* animal, it would be supposed that they should be as good glycogen formers as glucose. The reports in the literature are very much at variance with each other and none of them permits of any such conclusion as this. Pflüger and Junkersdorf (2) found that the liver glycogen increased when normal fasted rats were fed glycine. Wilson and Lewis (3) made observations on the liver glycogen of rats 1, 4, and 16 hours after the administration of these compounds and concluded that, while *dl*-alanine caused a rapid deposition of liver glycogen, glycine produced no glycogen. Hodgson (4) concluded that alanine does not readily form glycogen, while Reid (5) found that alanine forms glycogen easily, but concluded that glycine does not do so. The latter's conclusions were based upon observations made 4 hours after feeding, a point of interest in connection with our results. Butts *et al.* (6), as a result of observations made 8 hours after their administration, concluded that glycine is a fair glycogen former but that *dl*-alanine is a much better one.

There is one important point which previous investigators, with the exception of Wilson and Lewis (3), have not mentioned and that is the question of the *rate of formation of glycogen*. It would be very easy to conclude that two substances which formed equal amounts of glycogen were not of equal effectiveness as glycogen formers, if the rate of formation was different and sufficient observations were not made to measure the peak in the amount formed by each substance. Experiments carried out for

other purposes brought this possibility for glycine and alanine to our attention. These data and the interesting results reported by Luck (7) prompted us to undertake the experiments described here. He found that the administration of equivalent amounts of glycine and *dl*-alanine to rats increased in the same measure the amino acid content of the systemic blood and that, while alanine produced no change in the amino nitrogen content of either liver or muscle, glycine provoked a great increase of the amino nitrogen in both these tissues, which persisted for many hours. This of course suggests that the action of glycine proceeds much more slowly than that of alanine and hence that its influence on glycogen formation would be much slower.

EXPERIMENTAL

Our experiments were carried out on female rats 59 to 66 days old. They weighed from 91 to 136 gm. and the groups averaged 108 to 121 gm. in weight. They were fasted for 48 hours after receiving the stock diet and the solutions administered in the amount of 1 cc. per sq.dm. of body surface. Body surface was calculated from the gross body weight by the formula of Carman and Mitchell (8). The glucose and *dl*-alanine were Pfanstiehl's best preparations and the glycine used was Squibb's product. The glucose solution had a concentration of 1 mole, the glycine of 3 moles, and the *dl*-alanine of 2 moles per liter, so that the carbon equivalents of the three solutions were the same. The solutions were administered by stomach tube, and at intervals of 2 hours three groups of five rats each, one for each substance, were killed and liver glycogen determined by the method of Good *et al.* (9).

Results

The liver glycogen concentration increased to a maximum of 2.00 per cent after glucose feeding, 2.36 per cent after glycine, and 1.96 per cent after *dl*-alanine. The highest concentrations of glycogen in the three series of groups is reached after glycine feeding, which has heretofore had the reputation of being a very inferior glycogen former, if it formed any at all. The differences in the amount of glycogen formed are shown very well in Fig. 1. These experiments are not designed to determine whether or not

the larger amount of glycogen deposited after glycine feeding than after glucose or *dl*-alanine is significant. Although there is no great difference in the amount of glycogen formed, there are great differences in the rate of glycogen formation. Glucose reaches its peak at 6 hours and the *dl*-alanine only 4 hours later. At the peak of glycogen formation from glucose the glycine has not even begun to cause a measurable glycogen synthesis and the peak of glycogen deposition in the liver, due to feeding this

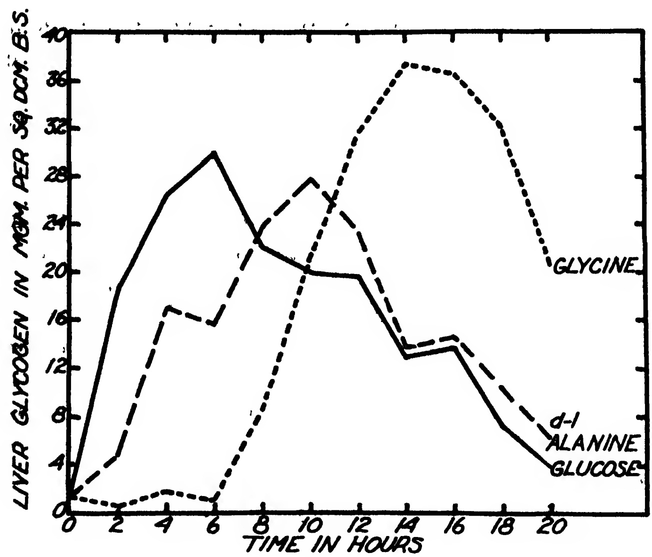

FIG. 1. Effect of feeding glucose, glycine, and *dl*-alanine on glycogen deposition in the liver, measured in mg. per sq.dm. of body surface.[1]

amino acid, is not reached until 8 hours later. It is easy to see from the data presented here the reason for the diverse conclusions reached in regard to the glycogen-forming efficacy of the compounds we have considered when observations were made at a single point.

[1] The detailed data and the averages used for the preparation of the graphic presentation of the results in Fig. 1 have been deposited with the Auxiliary Publication Service of American Documentation Institute, Washington, D.C., as supplementary material, Document No. 1319.

DISCUSSION

The differences in the rate of glycogen deposition in the liver might be related to differences in the rate of absorption from the intestine of glucose, glycine, and *dl*-alanine. Voegtlin *et al.* (10) raised this possibility in connection with the better protection against insulin hypoglycemia afforded by alanine than by glycine. The fact that glucose is absorbed more rapidly than alanine (11) might account for a part but not all of the difference in their relative rates of glycogen formation. The slower absorption of glycine than of *dl*-alanine or glucose (11) could hardly contribute very much to our results, for absorption is complete (11, 6) long before marked glycogen deposition occurs.

We have assumed throughout this text that the liver glycogen which was formed with the various feedings came by direct conversion of the substance fed. This is the most reasonable assumption but for glycine it is difficult from the purely chemical point of view to picture such a transformation. This led Dakin (12) to be "inclined to believe that a direct conversion of the carbon of glycine in glucose does not take place but rather that it causes a disturbance in the normal equilibrium existing between the amino-acids or peptides in the body tissues with the result that other amino-acids, capable of furnishing glucose (*e.g.* alanine) are set free." Reid's results (5) on the effect of glycine on the protein catabolism as measured by nitrogen and sulfur excretion in dogs would lend weight to this possibility.

SUMMARY

1. The amount of glycogen deposited in the livers of rats is not greatly different when equivalent amounts of glucose, glycine, and *dl*-alanine are fed. Maximum hepatic glycogen concentrations of 2.00, 2.36, and 1.96 per cent respectively are reached after glucose, glycine, and *dl*-alanine.

2. The rate of hepatic glycogen deposition is very different after glucose, *dl*-alanine, and glycine feeding. Glucose forms glycogen the most rapidly, reaching the peak in 6 hours. 10 hours after *dl*-alanine feeding the liver glycogen is at its height. After glycine feeding, liver glycogen is at first formed very slowly and the highest liver glycogen concentration is not reached for 14 hours.

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CYCLIZATION OF VITAMIN A_2*

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Because the absorption spectra of vitamin A_2 and its derivatives have bands located at wave-lengths from 5 to 80 mμ longer than those in the spectra of vitamin A_1 and its corresponding derivatives, it is generally believed that the structural formula for vitamin A_2 has six double bonds in conjugation instead of the five in the formula for vitamin A_1.

Gillam *et al.* (1938) showed that the formula for vitamin A_2 must contain the β-ionone ring, and they suggested that vitamin A_2 has the same structural formula as vitamin A_1 but with the —CH_2OH group replaced by the group —CH=CH—CH_2OH. They found, contrary to the implications of this structure, that vitamin A_2 could not be separated from vitamin A_1 by chromatographic adsorption or molecular distillation.

By the elimination curve technique, Gray (1939) found that the temperature of distillation of vitamin A_2 differed from that of vitamin A_1 by only 3° instead of the 10° that the distillation temperature of an anthraquinone dye differed from that of its homologue containing 2 less carbon atoms. Karrer, Ruegger, and Geiger (1938) synthesized a material which probably had the structure for vitamin A_2 proposed by Gillam *et al.* except for an additional methyl group. This material did not give the color reactions for vitamin A_2, although it had been shown that alkyl groups so located would not interfere with the optical properties of other carotenoids.

A reaction which promised to furnish more information on the difference between the structures of the two vitamins was their cyclization.

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When vitamin A_1 is treated with dry alcoholic hydrogen chloride, it is cyclized (Embree, 1939; Edisbury *et al.*, 1932) to yield cyclized vitamin A_1, a material which has absorption bands at 350, 368, and 389 mμ and gives a blue color with $SbCl_3$ that is very similar to that given by unaltered vitamin A_1. The cyclized vitamin A_1 is much less readily adsorbed chromatographically by alumina than are other products formed by cyclization of a rich vitamin A concentrate. Preparations with a value of $E^{1\%}_{1\,\text{cm.}}$ (368 mμ) equal to 2370 have been prepared.

Edisbury *et al.* (1938) mentioned that an extract from goldfish eyes (vitamin A_2) when treated with alcoholic HCl gave a substance which had ultraviolet absorption bands like those of cyclized vitamin A. The cyclization of a perch liver oil extract will be described below.

3 gm. of oil extracted from several livers of perch (*Perca flavescens*) were saponified, yielding 0.416 gm. of unsaponifiable matter. This material, an orange solid, had a value for $E^{1\%}_{1\,\text{cm.}}$ (340 mμ) of 8.88 in the ultraviolet region, and the blue color developed with $SbCl_3$ in chloroform had a value for $E^{1\%}_{1\,\text{cm.}}$ (620 mμ) of 7.77 and a value for $E^{1\%}_{1\,\text{cm.}}$ (690 mμ) of 19.9 in the visible range of the spectrum.

The unsaponifiable matter, 0.345 gm., was dissolved in alcohol and cyclized by making the solution N/30 with HCl and allowing it to stand at room temperature for 15 minutes. The solution was then neutralized with aqueous NaOH, diluted with water, and extracted with ether. From the ether extracts a residue of 0.344 gm. was obtained. This cyclized material had, as has cyclized vitamin A_1, an absorption spectrum (Fig. 1) with maxima at 350, 368, and 390 mμ. The value for $E^{1\%}_{1\,\text{cm.}}$ (368 mμ) of this material was 12.5. Despite the similarity of its ultraviolet absorption spectrum, this material cannot be identical with cyclized vitamin A_1, because its blue color developed with $SbCl_3$ has a maximum absorption at 690 mμ like that of unchanged vitamin A_2. Values for $E^{1\%}_{1\,\text{cm.}}$ (620 mμ) and $E^{1\%}_{1\,\text{cm.}}$ (690 mμ) were 6.3 and 15.4, respectively.

A petroleum ether solution of a portion of the cyclized material was passed through an adsorption tube filled with aluminum oxide (Brockman). Solvent was passed through the tube until the main band of the adsorbate had been moved about half-way

down the tube. The contents of the tube were divided into six fractions, three being washed out of the tube and the other three being removed with the adsorbing material.

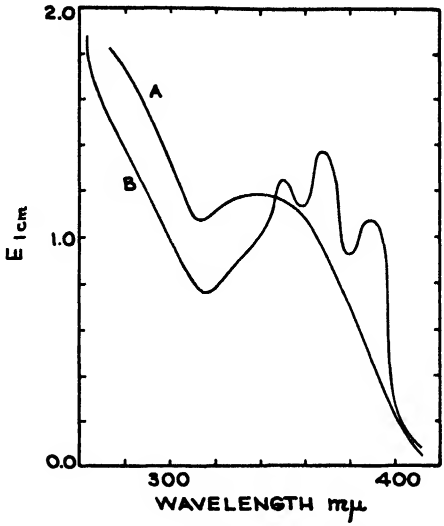

FIG. 1. Ultraviolet absorption spectra of the unsaponifiable fraction of perch liver oil before and after cyclization. Curve A, before cyclization, $E^{1.664\%}_{1\,cm.}$; Curve B, after cyclization, $E^{1.376\%}_{1\,cm.}$.

TABLE I

Chromatographic Analysis of Cyclized Perch Liver Oil Concentrate

Fraction No.	Weight	Description
	gm.	
1	0.0755	Colorless solution washed through with petroleum ether
2	0.0165	Yellow material washed through with petroleum ether
3	0.0045	" " " " " 1% EtOH in petroleum ether
4	0.0015	Material in tube below main band
5	0.0380	Main band, orange
6	0.1210	Material in tube above band (proved to be white solid upon removal of solvent)

The solvent was removed from each fraction under nitrogen. Table I describes the appearance of each fraction. The fractions were weighed and the ultraviolet absorption and $SbCl_3$ color were

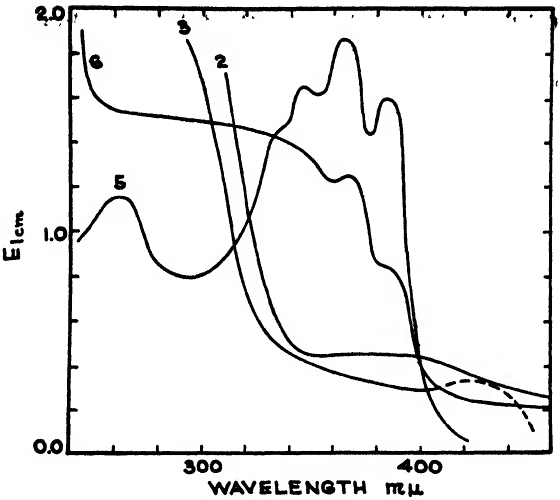

FIG. 2. Ultraviolet absorption spectra of the chromatographic fractions of the cyclized unsaponifiable residue of perch liver oil. The concentration of each fraction is as follows: Fraction 2, 0.132 per cent; Fraction 3, 0.018 per cent; Fraction 5, 0.0304 per cent; Fraction 6, 0.968 per cent.

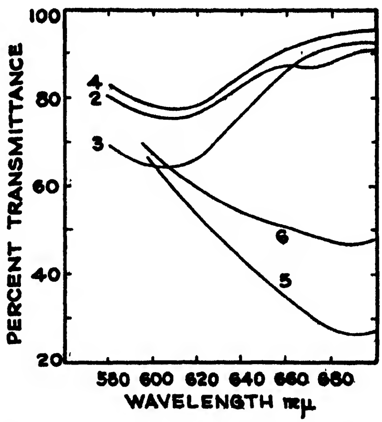

FIG. 3. Transmission spectra of the $SbCl_3$ reaction products of the chromatographic fractions of the cyclized unsaponifiable residue of perch liver oil. The measurements were made with the Hardy recording spectrophotometer as described by Embree (1939). The concentration of each fraction is as follows: Fraction 2, 0.405 per cent; Fraction 3, 0.00965 per cent, Fraction 4, 0.0103 per cent, Fraction 5, 0.00725 per cent; Fraction 6, 0.22 per cent.

immediately examined. The ultraviolet absorption spectra are shown in Fig. 2, and portions of the absorption spectra for the $SbCl_3$ blue color are shown in Fig. 3.

The optical data are summarized in Table II. Fraction 1 was a colorless oil which gave no $SbCl_3$ color and may have consisted mainly of hydrocarbons. Fraction 2 appeared to contain cyclized vitamin A_1, since its $SbCl_3$ blue color had the maximum absorption at 610 mμ, and it was not strongly enough adsorbed on alumina to be vitamin A_1. The ultraviolet absorption spectrum showed only general absorption in the region where the absorption of cyclized vitamin A_1 should be. The general absorption,

TABLE II

Optical Properties of Fractions from Cyclized Perch Liver Oil Concentrate

Material	$E^{1\%}_{1\,cm.}$ (620 mμ)	$E^{1\%}_{1\,cm.}$ (690 mμ)	$E^{1\%}_{1\,cm.}$ (368 mμ)
Unsaponifiable matter of perch liver oil	7.77	19.9	7.13
After cyclization	6.3	15.4	12.5
Fraction 2 from chromatographic separation of cyclized material	3.2*	1.23	3.3
Fraction 3	18.2*	4.25	19
" 4	11.4*	2.04	Not enough material
" 5	38.5	87.0	61
" 6	1.04	1.45	1.3

* The maximum was actually at 610 mμ.

however, was high enough to cover up the cyclized vitamin A_1 bands, since Embree (1939) showed that for cyclized vitamin A_1 the value for $E^{1\%}_{1\,cm.}$ (620 mμ) is higher than that for $E^{1\%}_{1\,cm.}$ (368 mμ), while for Fraction 2 the value for $E^{1\%}_{1\,cm.}$ (610 mμ) is lower than that for $E^{1\%}_{1\,cm.}$ (368 mμ). Fractions 3 and 4 seem to have contained the same material that was in Fraction 2.

Fraction 5, the orange material that was strongly adsorbed, had ultraviolet absorption bands at the same wave-length as those of concentrates of cyclized vitamin A_1, but the $SbCl_3$ blue color for this material had its maximum absorption at 690 mμ. The blue color was not due to the presence of enough vitamin A_2 to cover up the absorption due to cyclized vitamin A_1, since the latter, if

present in enough quantity to give a value of 61 for $E^{1\%}_{1\,cm.}$ (368 mμ), would have had a value of $E^{1\%}_{1\,cm.}$ (620 mμ) of about 83. We must conclude that the chromogenic material in Fraction 5 was cyclized vitamin A_2. Cyclized vitamin A_2 seems to have an ultraviolet absorption spectrum with bands at the same wavelength as those in the spectrum of cyclized vitamin A_1. However, it is much more strongly adsorbed on aluminum oxide than cyclized vitamin A_1, and with $SbCl_3$ it develops the blue color characteristic of vitamin A_2.

Fraction 6 seems to have consisted mainly of sterols with some cyclized vitamin A_2. The recovery from the chromatographic absorption was 75 per cent with respect to the absorption of light at 368 mμ by the fractions, or 79 per cent with respect to the absorption of light at 690 mμ by the $SbCl_3$ reaction products of the fractions.

The main chromogenic group of the atoms in cyclized vitamin A_1 or A_2, since their absorption maxima have the same wavelength, apparently does not include that part of the molecule which distinguishes vitamin A_1 from vitamin A_2. The conjugated double bond chain appears to have been broken by the cyclization reaction. Upon reaction with $SbCl_3$, the distinguishing part of the molecule becomes optically effective again, since the $SbCl_3$ reaction products of the cyclized vitamins A have the characteristic absorption bands of the corresponding vitamins A.

The fact that the cyclized vitamin A_2 is more strongly adsorbed than cyclized vitamin A_1 indicates that a method involving cyclization might be developed for estimating the contamination of one of the vitamins A by the other, or perhaps the quantitative estimation of the amount of both vitamins A. For example, the ratio of vitamin A_2 to vitamin A_1 in the perch liver oil can be calculated if several reasonable assumptions are made.

The first assumption is that there was the same per cent yield for the cyclization of each vitamin in the perch liver oil extract. The second assumption is that a value for $E^{1\%}_{1\,cm.}$ (368 mμ) will correspond to the same molecular concentration of either cyclized vitamin. This latter assumption is probably correct, since the ultraviolet absorption must be due to the same chromophoric group. However, in some of the fractions the ultraviolet absorption is obscured, so that the quantity of cyclized vitamin present

must be estimated by the $SbCl_3$ reaction product. To do this we have assumed that the ratio of the value for $E^{1\%}_{1\,cm.}$ (690 mμ) to the value for $E^{1\%}_{1\,cm.}$ (368 mμ) for a preparation of cyclized vitamin A_2, free from interfering substances, is 87:61 (the value of this ratio for Fraction 5), and we have also assumed that the ratio of the value for $E^{1\%}_{1\,cm.}$ (620 mμ) to the value for $E^{1\%}_{1\,cm.}$ (368 mμ) for a preparation of cyclized vitamin A_1 free from interfering substances is 1800:1318 (the value of this ratio found by Embree (1939)).

The amounts of cyclized vitamins A_1 and A_2 calculated as the product of $E^{1\%}_{1\,cm.}$ (368 mμ) times the weight of the fractions are

TABLE III

Yield of Cyclized Vitamin Fractions

Fraction No.	$E^{1\%}_{1\,cm.}$ (368 mμ)	$E^{1\%}_{1\,cm.}$ (368 mμ) $\times$ weight
2	2.4 (Calculated)	0.040
3	13.3 "	0.060
4	8.4 "	0.013
Total cyclized vitamin A_1		0.113
5	61.0	2.32
6	1.0 (Calculated)	0.12
Total cyclized vitamin A_2		2.44

shown in Table III. It is found that the ratio of the number of molecules of cyclized vitamin A_2 to those of cyclized vitamin A_1 is 2.44:0.113 or 22.5:1. This presumably is the ratio of the molecules of vitamin A_2 to those of vitamin A_1 in the perch liver oil. The equation suggested by Wald (1939) for the calculation of the ratio of vitamin A_2 to vitamin A_1 gives a value of 0 for the amount of vitamin A_1 present, which is well within the limits of error that he claimed for such an estimation.

An experiment has also been performed on the chromatographic separation of a mixture of a solution of chromatographically pure cyclized vitamin A_1 and a solution of chromatographically pure cyclized vitamin A_2. The recovery was good, being 92 per cent for cyclized vitamin A_1 and 93 per cent for the vitamin A_2 derivative.

The most doubtful step in this analysis of vitamins A_1 and A_2 from a quantitative point of view is the cyclization process. Our experience has indicated that the yield of the cyclized vitamin is the same for each species, however.

SUMMARY

Vitamin A_2 can be cyclized by the same methods which are used to cyclize vitamin A_1.

Cyclized vitamin A_2, like cyclized vitamin A_1, has an ultraviolet absorption spectrum with maxima at 350, 368, and 390 mμ.

The antimony trichloride reaction product of cyclized vitamin A_2, like that of vitamin A_2, has an absorption spectrum with a maximum near 690 mμ.

Cyclized vitamin A_2 is more strongly adsorbed by alumina than is cyclized vitamin A_1.

A method has been outlined for the estimation of the relative amounts of vitamins A_2 and A_1 by the separation of their cyclized derivatives.

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CHOLINE METABOLISM

II. THE INTERRELATIONSHIP OF CHOLINE, CYSTINE, AND METHIONINE IN THE OCCURRENCE AND PREVENTION OF HEMORRHAGIC DEGENERATION IN YOUNG RATS

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A new dietary deficiency in young rats which was prevented by choline was described in the first papers of this series (1). This deficiency was characterized by a severe hemorrhagic degeneration of the kidneys. The experiments demonstrated that choline was an indispensable dietary factor for the young rat and that it was required for the maintenance of the integrity of renal and other tissues as well as for the prevention of the deposition of fat or cholesterol in liver tissue (2).

In 1929 Cox, Smythe, and Fishback (3) reported an unexplained nephropathogenic action of cystine in rats on a purified ration to which cystine was added. Cox and Hudson (4) and others (5) concluded that an unrecognized factor in the vitamin B complex was concerned with this toxic action of cystine. Our experiments have demonstrated that the factor which prevents the injurious effect of cystine is choline and that methionine has a choline-like action (6). This same opposition of cystine to choline and to methionine has been observed in experiments on the production of fatty livers in older rats. Beeston and Channon (7) found that cystine increased the deposition of liver fat and Tucker and Eckstein (8) noted that methionine exhibited a lipotropic activity. The present paper deals with the interrelationship of choline and the two sulfur amino acids in the production and prevention of hemorrhagic degeneration in young rats.

EXPERIMENTAL

Male rats, 38 to 42 gm. in weight and averaging 24 days of age, were placed in raised cages and fed the experimental diets *ad*

libitum. At the end of the experimental period the rats were killed by decapitation. The liver, thymus, spleen, and kidneys were removed at once and weighed. The latter three organs were dried to constant weight in an oven at 105°. Total chloroform-soluble substances in the liver were determined by the method described by Channon, Platt, and Smith (9). The term "liver fat" in Tables III to VI refers in every case to this fraction. The components of the basal ration were the same as those described in Paper I ((1) p. 567).

Liver fat is expressed in the tables as the actual weight of chloroform-soluble substances, as its per cent in liver tissue, and as the ratio obtained by dividing the actual weight by the expected weight of the chloroform-soluble fraction of the livers of normal control rats. The weights of the liver, thymus, spleen, and kidneys are expressed as actual weights and as "per cent of normal" values. The latter values indicate the comparison between the weights of these organs in experimental and normal rats. It was previously shown that in the young male rats of this colony the liver weight was 4.31 per cent of the body weight and that there were 1.73 mg. of liver fat per gm. of body weight (1). Values for the fresh and dry weights of the thymus, spleen, and kidneys of normal male rats are shown in Tables I and II. Curves were drawn from these data so that the expected organ weights of experimental animals could be read directly.

Results

Effect of Choline in Prevention of Toxic Action of Cystine—The effect of choline in supplementing a low choline basal diet and the same diet plus 0.3 and 0.5 per cent of cystine is shown in Tables III and IV. Table III shows the basal diet, the additions of choline and of cystine, the occurrence of renal hemorrhagic degeneration, and the deposition of liver fat. Table IV shows the changes in the weights of the thymus, spleen, and kidneys of the same groups of rats recorded in Table III.

The basal diet without added cystine (Group 1, Table III) was moderately toxic. Comparison of the results with the basal diet (Group 1), basal diet plus 0.3 per cent of cystine (Group 6), and the basal diet plus 0.5 per cent of cystine (Group 8) confirmed the toxic action of cystine. The addition of cystine increased

the incidence of renal lesions, the deposition of liver fat, and the weights of the spleen and kidneys and decreased the weight of the thymus. These changes are characteristic of increased choline deficiency (1). Except for one rat in a group of twenty, the

TABLE I

Relation between Body Weight and Fresh Weight of Thymus, Spleen, and Kidneys in Young Normal Male Rats (Stock Diet)*

No. of rats	Body weight		Thymus weight		Spleen weight		Kidney weight (per pair)	
	Range	Average	Average	Per cent of body weight	Average	Per cent of body weight	Average	Per cent of body weight
	gm.	*gm.*	*mg.*		*mg.*		*mg.*	
20	43-49	45.7	182	0.398	207	0.454	555	1.215
40	50-62	55.2	227	0 412	306	0.555	654	1.184
40	63-74	68.2	271	0.398	429	0.629	752	1.102
20	75-83	77.8	302	0.388	563	0.724	846	1.088

* Purina Dog Chow.

TABLE II

Relation between Body Weight and Dry Weight of Thymus, Spleen, and Kidneys in Young Normal Male Rats (Stock Diet)

No. of rats	Body weight		Thymus weight		Spleen weight		Kidney weight (per pair)	
	Range	Average	Average	Per cent of body weight	Average	Per cent of body weight	Average	Per cent of body weight
	gm.	*gm.*	*mg.*		*mg.*		*mg.*	
28	46-54	50.0	42.7	0.085	49.1	0.098	124.0	0.248
15	56-65	61.8	52.3	0.085	82.6	0.134	155.4	0.251
32	66-75	70.8	57.4	0.081	103.5	0.146	169.9	0.240
19	76-89	81.3	62.7	0.077	122.8	0.151	191.6	0.235
31	43-74	55.0	45.6	0.083	63.8	0.116	144.2	0.262

addition of 0.25 mg. of choline chloride per gm. of food prevented the signs of hemorrhagic degeneration but only slightly diminished the fatty liver effect (Group 3). 3 times as much choline were required to protect equally the rats fed the basal ration plus 0.5 per cent of cystine (Group 11). Although an evaluation of the quantitative aspects of this opposition of cystine to choline was not

possible from these data, it was evident that the addition of an extra 0.5 mg. of choline chloride per gm. of food was necessary to offset the inclusion of 5 mg. of cystine per gm. of food. In this experiment 1 molecular equivalent of choline chloride compensated for approximately 6 equivalents of cystine.

TABLE III

Relation of Cystine and of Choline to Deposition of Liver Fat and to Appearance of Renal Lesions in 40 Gm. Male Rats during 10 Day Experimental Period on Low Choline Diet

Group No. and diet	No. of rats		Final body weight (average)	Per cent of rats with renal lesions	Cystine added to diet	Choline chloride added per gm. food	Liver		Liver fat		
	Start	End					Weight (average)	Per cent of normal	Weight (average)	Per cent of liver	Ratio of experimental to normal
			gm.		*per cent*	*mg.*	*gm.*		*mg.*		
1-BF3	40	37	54	60	0.0	0.00	3.71	159	1080	29.1	11.6
2-BF36	20	20	56	30	0.0	0.13	3.47	144	1131	32.7	11.7
3-BF35	20	20	58	5	0.0	0.25	3.34	133	925	27.7	9.2
4-BF40	10	10	61	0	0.0	0.75	2.90	110	281	9.7	2.7
5-BF41	10	10	64	0	0.0	1.00	2.83	103	246	8.7	2.2
6-BF5	21	18	53	86	0.3	0.00	3.81	167	992	26.1	10.8
7-BF7	20	20	62	0	0.3	5.00	3.27	122	141	4.3	1.3
8-BF18	20	16	52	95	0.5	0.00	4.22	188	1158	27.4	12.9
9-BF33	20	16	56	80	0.5	0.25	4.36	181	1248	28.6	12.9
10-BF34	20	20	59	65	0.5	0.50	4.12	163	1249	30.3	12.2
11-BF37	20	20	69	5	0.5	0.75	4.15	140	1019	24.5	8.5
12-BF38	20	20	65	0	0.5	1.00	3.34	119	426	12.8	3.8
13-BF39	10	10	69	0	0.5	2.00	3.39	114	195	5.8	1.6

Diet—Casein 15, lard 35, salt mixture (Hawk and Oser (10)) 4, calcium carbonate 1, agar 2, cod liver oil 5, yeast 6, cystine and choline as indicated, sucrose to 100.

The results of Tables III and IV demonstrated the wide-spread effects of the hemorrhagic degeneration. Four animals in each of Groups 8 and 9 died before the end of the experimental period. The fact that such acute signs of choline deprivation appeared within less than 10 days indicated that no adequate stores of choline were available. The parallel changes in fresh and dry weights of the thymus, spleen, and kidneys showed that the

tissue effects were not due merely to variations in water content. The autopsy of over 500 rats used in the experiments reported in this paper indicated that the change in the kidney weight is an accurate measure of the extent of the choline deficiency. The deposition of liver fat was a less accurate index, because the weight and fat content of the livers of the most severely affected rats

TABLE IV

Relation of Cystine and of Choline to Weights of Thymus, Spleen, and Kidneys in 40 Gm. Male Rats During 10 Day Experimental Period on Low Choline Diet

For other data refer to Table III.

Group No. and diet	Thymus				Spleen				Kidneys (per pair)			
	Fresh		Dry		Fresh		Dry		Fresh		Dry	
	Weight (average)	Per cent of normal	Weight (average)	Per cent of normal	Weight (average)	Per cent of normal	Weight (average)	Per cent of normal	Weight (average)	Per cent of normal	Weight (average)	Per cent of normal
	mg.		*mg.*		*mg.*		*mg.*		*mg.*		*mg.*	
1-BF3	143	65	32	70	257	87	54	89	800	125	165	122
2-BF36	176	77	39	81	269	86	60	90	635	96	132	94
3-BF35	194	82	43	88	219	66	49	68	565	84	121	84
4-BF40	224	91	47	91	314	87	70	87	598	86	127	83
5-BF41	241	94	51	94	260	67	57	65	631	88	137	86
6-BF5	115	52	25	56	321	113	71	122	927	143	190	144
7-BF7	264	106			291	79			685	97		
8-BF18	102	48	22	50	357	130	79	144	1003	162	196	151
9-BF33	114	50	25	52	437	140	96	143	861	131	168	120
10-BF34	175	73	38	76	406	119	91	121	814	119	163	110
11-BF37	232	85	49	88	346	79	74	74	678	89	144	86
12-BF38	245	94	52	96	332	83	73	81	626	86	133	83
13-BF39	277	102	57	102	418	95	91	91	687	90	147	88

were markedly decreased owing to their poor nutritional state. It has frequently been observed in these experiments that a large fatty liver could be diminished in size and fat content either by supplying adequate amounts of choline or *by further decreasing the choline intake so that hemorrhagic degeneration occurred.* Maximum fatty livers were produced on diets which contained enough choline to present the renal pathology.

Choline-Like Action of Methionine—The protective action of

both choline and methionine was illustrated by the results on Groups 12, 13, and 14 in Table V. Group 12 was fed a toxic basal diet. The marked hemorrhagic enlargement of the kidneys

TABLE V

Relation of Dietary Protein and of Methionine to Deposition of Liver Fat, to Occurrence of Hemorrhagic Degeneration, and to Weight of Kidneys in 40 Gm. Male Rats during 10 Day Experimental Period on Low Choline Diet. (Average Values per Group of Ten Rats)

Group No. and diet	Dietary protein				Final body weight	Liver		Liver fat			Kidneys (per pair)		Per cent of rats with renal lesions
	Total	Fibrin	Casein	Albumin		Weight	Per cent of normal	Weight	Per cent of liver	Ratio of experimental to normal	Weight	Per cent of normal	
	per cent	*per cent*	*per cent*	*per cent*	*gm.*	*gm.*		*mg.*			*mg.*		
1-BK1	5	0	0	5	42	2.15	118	486	22.6	6.7	450	87	0
2-BF1	5	0	5	0	44	2.63	139	743	28.2	9.8	460	86	0
3-BB	5	2	2	1	44	2.38	125	644	27.0	8.5	498	93	0
4-BT1	5	5	0	0	43	2.77	149	798	28.8	10.7	509	97	10
5-BF2	10	0	10	0	55	3.71	156	1240	33.4	13.0	583	90	10
6-BC	10	4	4	2	57	3.78	154	1059	28.0	10.7	685	103	40
7-BK2	15	0	0	15	63	4.33	159	1345	31.1	12.3	749	105	0
8-BF3	15	0	15	0	54	3.71	159	1080	29.1	11.6	800	125	60
9-BT3	15	15	0	0	50	3.76	174	1131	30.1	13.1	988	165	100
10-BX	15	4	8	3	54	3.71	159	894	24.1	9.6	1152	180	90
11-BH	15	6	6	3	53	4.13	180	949	22.9	10.3	1213	193	100
12-BF12	15	5	10	0	58	4.76	190	1422	29.9	14.2	997	148	70
13-BF9*	15	5	10	0	70	3.25	108	262	8.1	2.2	749	107	0
14-BF24†	15	5	10	0	68	3.28	112	324	9.9	2.8	695	93	0

The basal diet consisted of protein as indicated above, lard 35, salt mixture (Hawk and Oser) 4, calcium carbonate 1, agar 2, cod liver oil 5, whole dried yeast 6, and sucrose to 100.

* 10 mg. of *dl*-methionine added per gm. of food.

† 1 mg. of choline chloride added per gm. of food.

and the fatty liver were prevented if this basal diet was supplemented with either 10 mg. of *dl*-methionine per gm. of food (Group 13) or 1 mg. of choline chloride per gm. of food (Group 14). This same experiment was repeated with groups of seven rats. All of

the rats on the basal diet showed severe renal lesions, whereas the rats receiving the basal diet plus either methionine or choline were completely protected. It remains to be determined whether methionine may completely replace choline or whether it spares choline by decreasing the amount which is required, for instance, in preventing the injurious effect of cystine.

Relation of Dietary Protein to Choline Deficiency—In view of the opposing effects of cystine and methionine it was evident that the occurrence and severity of hemorrhagic degeneration on low choline diets would vary with the cystine and methionine content of the dietary protein. Table V shows some of the results which have been obtained when various mixtures of fibrin, casein, and dried egg albumin were used. The occurrence of hemorrhagic degeneration in some of these groups was of unusual interest, because these experiments were the first in which the new deficiency was observed on diets containing yeast. The results showed that, even though yeast supplied some choline, the deficiency appeared if the dietary protein was adequate for good growth and particularly if the protein mixture contained fibrin. Channon *et al.* (11) have reported that fibrin is less lipotropic than casein. With the exception of one rat in Group 4, renal lesions did not occur if the protein was fed at a 5 per cent level. All other combinations produced the deficiency, although only a few of the rats fed protein at a 10 per cent level were affected. There was no indication in these experiments that the effects of choline deficiency were due to inferior dietary protein. On the contrary the results emphasized the fact that the renal lesions were produced more readily if the dietary protein was adequate in amount and in composition for good growth.

The ratio of methionine to cystine in the dietary protein was not the only factor determining the incidence of hemorrhagic degeneration in these rats, although the results did suggest that proteins, high in methionine and low in cystine, were protective. The same protein mixture was fed at 5, 10, and 15 per cent levels to Groups 3, 6, and 11 (Table V) respectively. Renal lesions were absent in Group 3, moderate in Group 6, and very severe in Group 11. This variation in the effect of a protein fed at different levels was demonstrated by the results recorded in Table VI, which shows the effect of feeding casein at 5, 10, 15,

25, 35, 40, 45, and 47 per cent levels. The protein content of these diets was increased at the expense of the sucrose, so that no added carbohydrate was present in the ration fed Group 8. The

TABLE VI

Effect of High Casein Levels in Prevention of Deposition of Liver Fat and of Hemorrhagic Degeneration in 40 Gm. Male Rats during 10 Day Experimental Period on Low Choline Diet

Group No. and diet	Dietary		Cystine added to diet	No. of rats	Final body weight (average)	Liver		Liver fat			Kidneys (per pair)		Per cent of rats with renal lesions
	Casein	Sucrose				Weight (average)	Per cent of normal	Weight (average)	Per cent of liver	Ratio of experimental to normal	Weight (average)	Per cent of normal	
	per cent	*per cent*	*per cent*		*gm.*	*gm.*		*mg.*			*mg.*		
1-BF1	5	42	0	20	44	2.63	139	743	28.2	9.8	460	86	0
2-BF2	10	37	0	20	55	3.71	156	1240	33.4	13.0	583	90	15
3-BF3	15	32	0	40	54	3.71	159	1080	29.1	11.6	800	125	60
4-BF10	25	22	0	30	66	4.21	148	952	22.6	8.3	873	118	80
5-BF15	35	12	0	10	71	3.60	118	513	14.3	4.2	802	103	10
6-BF16	40	7	0	19	73	3.54	112	318	9.0	2.5	781	98	0
7-BF17	45	2	0	10	71	3.67	120	294	8.0	2.4	780	100	0
8-BF13	47	0	0	20	71	3.54	115	290	8.2	2.4	781	100	0
9-BF23*	47	15	0	20	70	3.38	112	191	5.7	1.6	823	107	0
10-BF30	25	21.5	0.5	20	53	3.88	170	957	24.6	10.4	964	155	80
11-BF32	40	6	1.0	20	70	3.12	103	211	6.8	1.7	777	101	0
12-BF31	46	15	1.0	20	71	2.75	90	131	4.8	1.1	792	102	0
13-BF19	46.5	0	0.5	10	66	3.10	109	236	7.6	2.1	753	102	0
14-BF27	45	0	2.0	10	67	3.28	114	204	6.2	1.8	834	112	0

The basal diet consisted of casein as indicated, sucrose as indicated, lard 35, cod liver oil 5, salt mixture (Hawk and Oser) 4, calcium carbonate 1, agar 2, and powdered yeast 6.

* 20 per cent of lard in basal diet.

maximum effects of choline deficiency were found if the casein level was 15 or 25 per cent (Groups 3 and 4).

Diets containing 40 per cent or more of casein were completely protective even if 2 per cent of cystine was added. The addition of 0.5 per cent of cystine greatly increased the toxicity of casein

fed at the 25 per cent level (Group 10). This protective action of the high casein diet was not due to the absence of carbohydrate, because the inclusion of 15 per cent of carbohydrate at the expense of fat did not alter the results (Group 9). These experiments in which the toxicity increased as the casein levels increased to 25 per cent and then decreased as the casein values were further increased demonstrated in a striking fashion that other factors than the methionine-cystine ratio of the dietary protein determined the requirement for choline.

DISCUSSION

The characteristic signs of hemorrhagic degeneration—enlarged hemorrhagic kidneys, enlarged spleen, shrunken thymus, and fatty liver—were all intensified by the addition of cystine and were prevented by the addition of methionine to the ration. It was concluded, therefore, that cystine and methionine in proteins are concerned with this new dietary deficiency and that the amounts of these two amino acids, in part, determine the requirement of the young rat for choline. Other factors such as dietary fat and cholesterol which also affect the requirement for choline will be discussed in Paper III of this series.

The demonstration that the injurious effect of cystine in a low choline ration is prevented by relatively small amounts of choline may be of importance in the explanation of the many reports in the literature of the damaging action of cystine and of various proteins on the kidneys. Lewis (12) for instance noted a severe nephritis in rabbits following the administration of cystine. Curtis and Newburgh (13) made similar observations on rats. Hartwell (14) found that rations containing edestin produced renal injuries. Certain of these investigations are being repeated in order to determine the possible rôle of choline under experimental conditions different from those reported in this paper.

The protection against hemorrhagic degeneration observed in these experiments on diets containing 40 per cent or more of casein was not due to small amounts of choline in the casein, because 15 and 25 per cent levels were more toxic than 5 and 10 per cent levels. Best and Channon (15) reported that an increase in the casein level above 5 per cent decreased the fatty liver effect in older rats. The protection on the high casein diet, especially the

protection in the presence of added cystine, gave further support to the conclusion that the choline requirement was not wholly dependent upon the cystine-methionine ratio of the protein. It is possible that cystine was incompletely absorbed from the alimentary tract or that intestinal putrefaction produced significantly greater amounts of choline on the high protein diets. Inasmuch as 83 to 90 per cent of the total sulfur of casein is present as methionine (16), it may be that the excess of methionine was responsible for the protection regardless of the cystine intake. The investigation of these and associated problems involving the relationship of choline and the two sulfur amino acids is being continued.

SUMMARY

1. The injurious effect of cystine on the kidneys of the rat has been confirmed.

2. Dietary cystine probably plays a rôle in the production of hemorrhagic degeneration in young rats on low choline diets.

3. Choline prevents hemorrhagic degeneration and the toxic effects of cystine.

4. Methionine has a choline-like action and prevents the appearance of renal lesions on a low choline diet.

5. Choline deficiency is, in part, dependent upon the composition and the amount of the dietary protein.

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CHOLINE METABOLISM

III. THE EFFECT OF CYSTINE, FAT, AND CHOLESTEROL ON HEMORRHAGIC DEGENERATION IN YOUNG RATS

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The rôle of choline in the prevention of hemorrhagic degeneration in young rats has been described in Paper I of this series (1). The deficiency was aggravated by dietary cystine and the toxic effects of cystine were alleviated by choline. Furthermore, it was demonstrated that methionine exhibited a choline-like action (2). The investigations of Best (3–6) and of Channon (6, 7) and their coworkers on fatty and cholesterol livers in older rats have emphasized the relation between choline, fat, and cholesterol. The metabolism of choline has been reviewed by Best and Ridout (8). The present paper deals with the occurrence on low fat diets of this recently recognized result of choline deficiency and with the effect of added fat, cystine, and cholesterol.

EXPERIMENTAL

The experimental procedures were the same as those used in Papers I and II (1, 2). The thymus, spleen, kidneys, and liver were weighed as in the previous experiments but the weights of the kidneys and liver, only, are reported in this paper. Liver fat in Tables I to III refers to total chloroform-soluble substance in the liver. The composition of the diets is shown in Tables I to III.

Results

Relation of Dietary Fat to Hemorrhagic Degeneration—The results in Table I showed that hemorrhagic degeneration could be produced on a low fat diet but that the deficiency was more severe

if the food mixture contained added fat. Furthermore, it was evident that the toxic action of cystine and the protective influence of a high casein level were independent of dietary fat. Previously these two effects were noted on diets containing 35 per cent of lard and 5 per cent of cod liver oil. The basal diet, AC24 (Table I), contained no added fat other than the 0.1 per cent of the fortified fish liver oil, Natola.[1] Ether-extracted yeast was used.

TABLE I

Relation of Dietary Fat to Deposition of Liver Fat and to Occurrence of Hemorrhagic Degeneration in 40 Gm. Male Rats during 10 Day Experimental Period

The basal diet, AC24, except as indicated, consisted of casein 15, salt mixture (Hawk and Oser (9)) 4, calcium carbonate 1, agar 2, Natola 0.1, ether-extracted yeast 5, and sucrose to 100.

Group No. and diet	No. of rats		Final body weight, average	Liver weight, average	Liver fat, average	Kidneys (per pair)		Per cent of rats with renal lesions	Dietary		
	At start	Final				Weight, average	Per cent of normal		Corn oil fatty acids	Lard	Cystine
			gm.	*gm.*	*mg.*	*mg.*			*per cent*	*per cent*	*per cent*
1-AC24	20	19	59	3.67	603	658	96	25	0	0	0.0
2-AC10	19	18	57	3.66	698	754	113	47	1	0	0.0
3-AC13	20	19	52	3.48	858	842	136	75	1	8	0.0
4-AC11*	10	10	62	2.73	121	663	94	0	1	0	0.0
5-AC30	20	14	60	4.41	798	874	127	95	0	0	0.5
6-AC31†	20	20	62	3.08	118	802	114	0	0	0	0.5

* 0.3 per cent of choline chloride added.

† Casein increased to 47 per cent.

Renal lesions appeared in 25 per cent of the rats on this diet (Group 1), even though the dietary protein was a protective protein with a high methionine and low cystine content. The addition of 1 per cent of corn oil fatty acids to this low fat basal ration caused an increase in the renal damage, as shown by the weight of the kidneys and the greater incidence of renal hemorrhage (Group 2). These effects were more pronounced if 8 per

[1] We wish to thank Parke, Davis and Company for the generous supply of Natola used in these experiments.

cent of lard was added to the diet in addition to the supplement of corn oil fatty acids (Group 3). The low fat ration was made very toxic by the addition of 0.5 per cent of cystine (Group 5). This result demonstrated that the mechanism producing renal injury due to cystine was not dependent upon the presence of dietary

TABLE II

Relation of Dietary Fat, Cholesterol, and Cystine to Deposition of Liver Fat and Occurrence of Hemorrhagic Degeneration in 40 Gm. Male Rats during 10 Day Experimental Period

Basal Diet AC17 consisted of casein 15, salt mixture (Hawk and Oser) 4, calcium carbonate 1, agar 2, powdered yeast 6, Natola 0.1, corn oil fatty acids 1, and sucrose to 100. Basal Diet BF3 was the same except that cod liver oil 5 and lard 35 replaced the Natola and corn oil fatty acids.

Group No. and diet	No. of rats		Final body weight, average	Liver weight, average	Liver fat, average	Kidneys (per pair)		Per cent of rats with renal lesions	Dietary		
	At start	Final				Weight, average	Per cent of normal		Lard	Cystine	Cholesterol
			gm.	*gm.*	*mg.*	*mg.*			*per cent*	*per cent*	*per cent*
1-AC23*	30	30	63	3.59	483	741	104	20	0	0.0	0
2-AC17	30	28	62	3.88	868	720	102	30	0	0.0	0
3-AC29*	20	20	62	3.78	881	694	98	20	1	0.0	0
4-AC14	30	30	56	3.91	888	838	127	60	8	0.0	0
5-AC20	33	28	61	4.34	805	947	136	80	0	0.3	0
6-AC26	30	22	55	3.63	925	854	131	85	8	0.3	0
7-AC15	30	26	57	3.64	715	763	114	55	0	0.0	1
8-AC25	30	20	55	3.76	1118	822	126	85	8	0.0	1
9-BF3	40	37	54	3.71	1080	800	125	60	35	0.0	0
10-BF11	30	17	50	3.69	994	898	150	85	35	0.0	1
11-BF21†	10	10	56	2.70	267	563	85	0	35	0.0	1

* Corn oil fatty acids omitted.
† 0.1 per cent choline chloride added.

fat. In Paper II (2) it was observed that a high fat diet containing cystine and 40 per cent, or more, of casein was protective against both the fatty liver and the renal damage. This was also found to be true for the high casein and low fat diet (Group 6). The livers of all of the rats on these low choline diets were fatty. Choline was effective in preventing the deposition of liver fat and the renal pathology (Group 4).

The effect of the addition of cystine, with and without added fat, was demonstrated by the results shown in Table II. The basal diet, AC17, contained 1 per cent of corn oil fatty acids and 6 per cent of whole dried yeast in place of the 5 per cent of ether-extracted yeast used in Diet AC24 (Table I). On this diet the results with Groups 1, 2, and 3 (Table II) showed that the presence or absence of 1 per cent of corn oil fatty acids or of 1 per cent of lard had little effect upon the occurrence of hemorrhagic degeneration. However, if the lard supplement was increased to 8 per cent (Group 4), the renal damage was as severe as that on the diet containing 35 per cent of lard (Group 9). The addition of 0.3 per cent of cystine to the basal low fat ration likewise increased the severity of the renal damage (Group 5) but the increased toxicity due to lard and cystine together did not appear to be additive (Group 6).

TABLE III

Relation of Length of Experimental Period to Deposition of Liver Fat and to Occurrence of Hemorrhagic Degeneration in 40 Gm. Male Rats on Low Choline Diet

The basal diet, AC20, consisted of casein 15, cystine 0.3, sucrose 70.6, corn oil fatty acids 1, salt mixture (Hawk and Oser) 4, calcium carbonate 1, agar 2, Natola 0.1, and whole dried yeast 6.

Experimental period	No. of rats		Final body weight, average	Liver weight, average	Liver fat		Kidneys (per pair)		Per cent of rats with renal lesions
	At start	Final			Weight, average	Ratio of experimental to normal	Weight, average	Per cent of normal	
days			*gm.*	*gm.*	*mg.*		*mg.*		
2	10	10	41	2.25	196	2.8	492	99	0
4	10	10	51	3.30	728	8.8	591	97	0
6	10	10	55	4.03	1002	10.6	732	112	80
8	10	8	61	4.34	1091	10.3	981	141	80
10	23	20	61	4.34	690	6.5	937	134	80

Table III shows the rapid onset of the effects of choline deficiency on a low fat ration containing 0.3 per cent of added cystine (Diet AC20). The deposition of liver fat was evident on the 2nd day and nearly maximal on the 4th day. Renal degeneration

was severe on the 6th day. The appearance of these signs may be further hastened by the addition of 8 per cent of lard to the diet (unpublished experiments).

Relation of Cholesterol to Hemorrhagic Degeneration—Table II also shows the effect of supplementing the low fat ration, Diet AC17, with cholesterol and with cholesterol and lard. Comparison of Groups 2 and 7 shows that a supplement of 1 per cent of cholesterol increased the damaging effect of the deficiency of choline on a low fat ration. If both cholesterol and lard were added to the diet, the renal injury was greater than that due to either supplement alone (Groups 4, 7, and 8). One-third of the rats in Group 8 died before the end of the 10 day period. It is possible that the more marked effect of cholesterol and lard together was due to the increased absorption of the cholesterol on the diet containing added fat. The effect of cholesterol in increasing the signs of choline deficiency was also evident in rats fed the high fat ration, Diet BF3 (Table II). The addition of 1 per cent of cholesterol resulted in a very marked hemorrhagic enlargement of the kidneys (Group 10). Only seventeen out of thirty rats survived on this diet. The toxic effect of cholesterol was prevented by choline (Group 11).

DISCUSSION

Supplements of cystine, fat, or cholesterol increased the severity of the hemorrhagic degeneration occurring in young rats on a low choline, low fat, and low cholesterol diet. These toxic effects were prevented by choline. The results reported in this and in previous papers (1, 2) suggest that hemorrhagic degeneration in young rats is a manifestation of the same deficiency which results in fatty and cholesterol livers in older rats on a low choline diet. However, the effect of choline deficiency in the young rat is not limited to a relatively non-injurious deposition of liver lipids. The kidney, particularly, undergoes an extremely rapid and acute hemorrhagic degeneration which may result in the death of the animal within a 10 day period. It has not yet been determined whether this new aspect of choline deficiency is another phase of abnormal lipid metabolism or whether a new rôle of choline in the animal body is involved. The discussion of the

significance of our findings must await completion of studies in progress on the chemical changes occurring in the tissues and body fluids of these rats.

SUMMARY

Hemorrhagic degeneration, which occurs in young rats on a low fat, low choline diet containing 15 per cent of casein, is increased in severity by the addition of cystine, lard, or cholesterol. The renal lesions appear in 6 days on diets containing added cystine. The toxic effect of these supplements is prevented by choline. Hemorrhagic degeneration does not occur on a low fat diet containing 47 per cent of casein and 0.5 per cent of cystine.

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WATER AND ELECTROLYTE CONTENT OF NORMAL AND HYDRONEPHROTIC KIDNEYS

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The specific purposes of the present study were (1) to determine the water content and electrolyte concentrations of normal dog kidneys and to utilize these data for a comparison of the right and left kidneys, and of the medulla and cortex, in normal animals; and (2) to determine the effect of deranged kidney function and structure on the water and electrolyte content of kidney.

The renal mass is divided into two equal parts, a right and a left kidney. That the weights of the kidneys of an animal are approximately equal has been established. Would the water content and electrolyte concentrations also be the same? What would be the relative distribution in the medulla and cortex? After values for normal animals were established, would a study of the electrolyte and water content of hydronephrotic kidneys show any relation between the chemical composition of the kidney and the structural and functional changes?

Physiological Procedures—This study includes analytical data on kidneys from the following groups: (1) the whole kidneys from twenty normal controls; (2) five normal controls, in each of which one whole kidney was analyzed and the other roughly dissected into medulla and cortex, which were analyzed separately; (3) fifteen hydronephrotic dogs, each having a single hydronephrotic kidney.

The normal dogs used in these experiments had been maintained under observation in metabolism cages for at least 3 weeks and were in excellent physical condition. The dogs with experimental hydronephrosis were also maintained in metabolism cages throughout the long experimental period (3 to 6 months).

All nephrectomies were performed under ether anesthesia. The kidney was brought outside of the split muscle layers, through an incision below and parallel to the left costal margin, and most of the fat was dissected from it. A clamp was applied to the pedicle and the organ was removed instantly. The kidney was at once split into halves, wiped with sterile absorbent gauze to remove all adherent blood and urine, and placed in a glass-stoppered weighing bottle. Just before the kidneys were weighed the capsule was removed from each half, and the tissue was wiped again and returned to the weighing bottle. The kidneys were weighed into silica beakers for water and fat analyses. Every kidney was treated identically.

When the medulla and cortex were to be analyzed separately, the halved kidney was placed on a tile and the medulla roughly dissected from the cortex. An exact dissection could not be made. The separated zones were placed in glass-stoppered weighing bottles and each treated as in the whole kidney analyses.

The damaged kidneys were obtained from dogs in progressive hydronephrosis, some of which were in uremia. Detailed methods for the production and diagnosis of hydronephrosis were presented in a previous paper (1). In this procedure, production of the hydronephrosis in one kidney is followed by removal of the normal kidney, so that the animal has a single damaged kidney with which to carry on all renal excretion.

Chemical Methods—The following determinations were carried out: water, neutral fat, chloride, sodium, potassium, calcium, and magnesium. All values were calculated for tissue on a fat-free basis. Unfortunately the amount of circulating blood in the kidneys analyzed could not be determined because samples of the wet tissue could not be sacrificed.

Water—Halves or quarters of decapsulated kidney (depending on the mass of the kidney) were placed in weighed silica beakers and their weights obtained by difference. The tissue was then minced with scissors, the scissors points rinsed with water, and the beakers placed in an oven at 103° until constant weight was attained (usually 72 hours were required). The sum of the weights of the dry tissue was subtracted from the sum of the weights of the wet tissue to give the water content of the whole kidney.

Neutral Fat—The dried tissue prepared for the water determina-

tions was covered with ethyl ether, and the beakers containing the material were placed in an empty glass desiccator with a ground glass cover. They were allowed to stand for about 3 hours, after which the ether was drawn off by a fine tipped pipette. This process was repeated. The kidney residue was then covered with petroleum ether (b.p. 40–60°) and the beakers were allowed to stand in the desiccator overnight. This extraction with petroleum ether was repeated until constant weight was reached. The sum of the weights was used to calculate the total weight of fat extracted and the percentage of fat in the whole kidney.

Preparation of Tissue for Electrolyte Determination—Unfortunately, the technique used to prepare skeletal muscle for chemical analyses could not be employed. When the lack of homogeneity of the kidney tissue, owing to structural differentiation into medulla and cortex, was considered, it was surmised that analytical results would deviate widely if random samples of minced tissue were used for the determinations. Experimentation proved this to be true. It was therefore necessary to work out a new technique for the treatment of kidney tissue.

Following the determinations of water and fat content, all of the portions of dry fat-free tissue from one kidney were transferred quantitatively to a special apparatus (2) and pulverized. The powdered tissue was swept with a brush ($1 \times 1\frac{1}{2}$ inches) into a large weighing bottle through a No. 20 copper sieve. Any particles remaining in the sieve were ground in an agate mortar until they could be sifted through. This method assured a homogeneous mixture of the kidney tissue. The weighing bottle containing the powdered tissue was kept in a desiccator over sulfuric acid, and aliquot samples of the powder were weighed for all analyses.

Chloride—Chlorides were determined by the wet ashing method of Van Slyke (3), and modified as follows: Approximately 150 mg. of the powdered tissue were weighed on a small filter paper (4.25 cm.) which was then folded and dropped into a 20×2.5 cm. digestion tube containing 5 cc. of water and 1 cc. of 0.075 N $AgNO_3$. The mixture was allowed to stand overnight; 3 cc. of concentrated nitric acid were then added and the material was digested. The results obtained by this method (4) agreed with those obtained by the Sunderman-Williams method (5). The

variable results which have been reported on the use of the wet ashing method for tissues with a high chloride content must be attributed to the errors unavoidable in random sampling. The concentrations of chloride differ so much in the medulla and the cortex of kidney that random sampling of wet tissue produces large errors.

Sodium and Potassium—For the determination of sodium and potassium, about 1.5 gm. of the kidney powder were weighed into a platinum dish and 5 cc. of 4 N H_2SO_4 then added. The dish was heated in an oven at 110° overnight and then ignited in a muffle furnace at 500°. Analyses were then carried out by the Butler-Tuthill method for sodium and by the Shohl-Bennett method for potassium, as described in detail in a previous paper (6).

Calcium and Magnesium—Approximately 2.0 gm. of the powdered tissue were weighed into a platinum dish and ignited at 550°. The residue was then dissolved in warm dilute HCl and transferred to a 10 cc. volumetric flask. The determinations were carried out on this solution.

For calcium determinations 3 cc. aliquots were used. The pH was carefully adjusted to between 4.2 and 4.4, with dilute ammonia, 1 drop of brom-cresol green being used as indicator. The calcium was then precipitated as calcium oxalate. After centrifugation, the precipitate was washed and dissolved in sulfuric acid for titration with potassium permanganate.

Magnesium was determined on 3 cc. aliquots by the method of Denis (7), with the following modifications. 3 cc. were placed in a 10 cc. volumetric flask and the pH adjusted to 4.2 to 4.4 as before. 1 cc. of saturated ammonium oxalate was added and the solution made up to volume and placed in the ice box overnight. The calcium oxalate was then centrifuged out, and the centrifugate used for magnesium determination. 4 cc. of the centrifugate were placed in a 15 cc. conical tube having a long slender point, and 1 cc. of 5 per cent ammonium acid phosphate and 1 cc. of dilute ammonia were added. The tube was allowed to stand in the ice box overnight and the magnesium ammonium phosphate was then collected by centrifugation and washed. The amount of magnesium was estimated from the colorimetric determination of phosphate by the method of Fiske and Subbarow (8).

Results

Normal Kidneys. Values for Whole Kidneys—The detailed results of the analysis of ten representative normal, whole kidneys are given in Table I, together with the mean values, with standard deviations, for twenty normal kidneys.

TABLE I

Water and Electrolyte Content of Normal Kidneys

The values are given per kilo of fat-free tissue.

Dog No.	H_2O	Fat	K	Na	Cl	Ca	Mg
	gm.	*gm.*	*mM*	*mM*	*mM*	*mM*	*mM*
505	795.5	31.1	60.6	89.1	77.8	2.19	6.20
508	796.9	27.1	64.6	79.6	65.7	2.30	5.80
511	803.0	15.6	62.8	78.8	68.8	2.01	6.10
514	792.3	26.1	60.8	84.1	70.9	1.93	5.82
G	801.2	17.9	59.3	79.9	65.2	2.01	6.10
R	809.4	28.0	57.1	83.8	65.7	1.96	5.30
E	805.3	15 6	59.1	80.9	64.0	2.07	5.48
H	807.6	18.5	54.1	88 0	67.6	2.03	5 91
512	802.9	15.8	48.2	89.1	67.7	2.95	4.70
C	809.3	12.3	55.7	85.5	66.5	3.40	5.83
20 normal kidneys							
Mean	802.2	19.7	58.3	82 6	67.7	2.16	5.7
σ*	5.6	9.0	4.8	5.8	5.3	0.53	0.5
Muscle from 20 normal dogs†							
Mean	765.0		82.1	32.4	21.5		
σ*	6.4		10.0	4.8	2.8		
Serum from 20 normal dogs†							
Mean	922.0		3.72	142.0	109.0		
σ*	6.0		0.4	4.1	1.7		

* Standard deviation.

† See (12).

It will be observed that the mean values for water and electrolyte contents were strikingly constant. The greatest deviation was found in the values for sodium and chloride. The percentage

of neutral fat was consistently low. The calcium values (2.16 mM per kilo) were similar to those found (9–11) for other mammalian kidneys. The magnesium content amounted to 5.7 mM per kilo, with a deviation of only 0.5 mM.

Values for Medulla and Cortex, and for Right and Left Kidneys—Data for two representative experiments carried out on whole left

TABLE II

Water and Electrolyte Content of Normal Right and Left Kidneys and of Roughly Dissected Medulla and Cortex

The values are given per kilo of fat-free tissue.

	Whole left kidney	Right kidney		
		Whole kidney (calculated)	Medulla	Cortex
Dog 505				
Total weight, *gm*	62.45	65.89	21.82	44.08
Water, *gm*	795	799	823	788
Fat, *gm*	31.1	30.9	42.3	25.4
Potassium, *mM*	60.6	60.3	49.1	65.7
Sodium, *mM*	89.1	88.4	124.9	70.7
Chloride, *mM*	77.8	77.4	117.1	58.0
Calcium, "	2.19	2.22	2.57	2.05
Dog 501				
Total weight, *gm*	58.26	58.04	17.63	40.41
Water, *gm*	803	800	825	790
Fat, *gm*	48	36	96	9.2
Potassium, *mM*	59.4	60.6	49.6	65.0
Sodium, *mM*	90.6	92.3	124 8	79.4
Chloride, *mM*	81.1	82.3	112.9	70.1
Calcium, "	3.20	3.33	3.43	3.50

kidneys and roughly dissected medulla and cortex from the opposite kidneys are presented in Table II. Water and electrolyte concentrations in the right and left kidneys of the same animal were approximately the same. Comparison of the medulla and cortex showed greater water, fat, sodium, and chloride values for the medulla than for the cortex; calcium concentrations were approximately the same; and the potassium concentrations were usually larger in the cortex than in the medulla.

Hydronephrotic Kidneys. Comparison of Hydronephrotic and Normal Values—The results obtained from the analysis of ten hydronephrotic kidneys are presented in detail in Table III, along with the means, with standard deviations, for fifteen kidneys. The variation in the concentrations of the constituents of these kidneys parallels the variation found in the degree of kidney damage in the hydronephrotic animals. In all cases approximately 50 per cent of the tissue of the single hydronephrotic kidney had been destroyed; but there was considerable variation in the relative amounts of medullary and cortical destruction and in the degree of hypertrophy in the residual tissue. In some cases there was some infection present; in others there was no gross or microscopic evidence of any infectious process.

TABLE III

Water and Electrolyte Content of Hydronephrotic Kidneys

The values are given per kilo of fat-free tissue.

Dog No.	H_2O	Fat	K	Na	Cl	Ca	Mg
	gm.	*gm.*	*mM*	*mM*	*mM*	*mM*	*mM*
68	836.8	8.50	59.9	85.9	58.3	5.47	5.44
To	841.5	2.0	49.4	65.3	42.2	2.78	
Do	823.9	2.0	53.4	76.6	55.1	2.59	5.44
Sp	819.2	9.0	60.7	65.1	57.8	3.14	6.90
R	837.4	6 8	62 2	76.3	61.6	2.55	5.24
Sn	817.8	12.5	54.0	77.3	56.3	4.93	6.05
W	832.5	1.3	59.0	75.6	52.4	3.82	5 30
J	831.6	0.6	59.5	87.8	41.2	4.08	5.78
Sc	844.7	13.8	45.4	88.6	52.3	2.03	5.70
T	832 2	1.7	58.4	82.4	61.6	5.32	4.51
15 hydronephrotic kidneys							
Mean	830.1	5.13	56.4	77.3	53.0	3.56	5.68
σ*. . . .	11.3	4.5	4.8	9.3	7.0	1.16	0.62

* Standard deviation.

DISCUSSION

In a previous paper Hastings and Eichelberger (12) presented analytical studies of skeletal muscle similar in scope to those

presented here for kidney. Assuming that the intracellular phase of skeletal muscle contains no chloride, it was possible to calculate the relative proportions of the intra- and extracellular phases of this comparatively simple system from the composition of the muscle and of the serum from the same animal.

It is obvious that, with even the same analytical data, it is not possible to make the same assumptions or calculations for kidneys. Not only is the system much more complex, with variations in structure and function within the kidney itself, but there is also the definite fact that certain cells of the kidney, engaged in the reabsorption of chloride from the glomerular filtrate, must contain chloride and sodium as well. Furthermore, the existence in the lumen of the nephron of an additional fluid phase, varying in composition as it passes down the renal tubules but attaining at times a concentration in sodium and chloride considerably higher than that of the plasma or intercellular or intracellular phases of the kidney, introduces additional complications. In spite of these uncertainties which make impracticable the quantitative interpretation of the data in the same manner as for skeletal muscle, it is possible to attain some tentative conclusions both for the normal kidney and for the kidney as altered by chronic hydronephrosis.

Normal Kidneys—Table I shows that the kidneys of normal dogs, as compared with skeletal muscle, are high in water, sodium, and chloride, and low in potassium. This suggests that the intracellular phase of the kidneys, as represented by the figures for potassium, is relatively low in amount. The values for sodium and chloride, however, are too high to be accounted for by the extra water, assuming that this water is extracellular and contains the concentration of sodium and chloride expected in intercellular fluids. The interpretation of the data is aided by reference to Table II, from which it will be seen that the differences from muscle are still further accentuated in the renal medulla but still exist in the cortex.

The concentration of chloride in the medulla is higher than the average for the plasma for normal dogs. Consequently, it must be assumed that part of the chloride is either in cells, or as appears more likely for the medulla, is in relatively high concentration in the contents of the collecting tubules and possibly also in the loops of Henle. The low concentration of potassium in the

medulla, together with the high water content, indicates the probability of a high proportion of extracellular fluids, a part of which apparently must be in the tubules.

Separation of the kidney into cortex and medulla brings the analytical data for the cortex closer to those of skeletal muscle, but the deviation in water, sodium, chloride, and potassium content still persists. In this case also it is doubtful whether this

TABLE IV

Comparison of Data for Normal and Hydronephrotic Animals

Mean indicates the arithmetical mean of the experimental values; σ, standard deviation; D, the difference between the means for hydronephrotic and normal kidneys; σd, the standard deviation of the difference; and P, the probability that the differences found are due to random sampling.

The values are given per kilo of fat-free tissue.

		Mean	σ	D	σd	P
		gm.	*gm.*	*gm.*	*gm.*	*per cent*
Water	Normal	802.2	5.6			
	Hydronephrotic	830.1	11 3	27.9	12.6	2.8
		mM	*mM*	*mM*	*mM*	
Chloride	Normal	67.7	5.3			
	Hydronephrotic	53.0	7.0	14.7	8.8	9
Sodium	Normal	82.6	5.8			
	Hydronephrotic	77.3	9.3	5.3	11.0	63
Potassium	Normal	58.3	4.0			
	Hydronephrotic	56.4	4.8	1.9	6.3	74
Calcium	Normal	2 16	0.53			
	Hydronephrotic	3.56	1.16	1.40	1.27	27
Magnesium	Normal	5.70	0.50			
	Hydronephrotic	5.68	0.62	0.02	0.8	99

deviation can be wholly accounted for by an increase in the intercellular fluid, and it is therefore necessary to assume that the analytical data either reflect fluid contained in the renal tubules and containing a relatively high concentration of sodium and chloride, or that the cells of the convoluted tubules, engaged in the reabsorption of chloride, add to the sodium and chloride concentrations; or that both of these are possibilities. In view of the increased complexity of the system, and of the increased number of variables, it is surprising that the data for whole

kidneys exhibit the degree of consistency portrayed in Tables I to III.

The data further indicate the necessity of analyzing whole kidneys. Since the distribution of water and electrolytes differs so distinctly in the medulla and cortex, and since the two zones

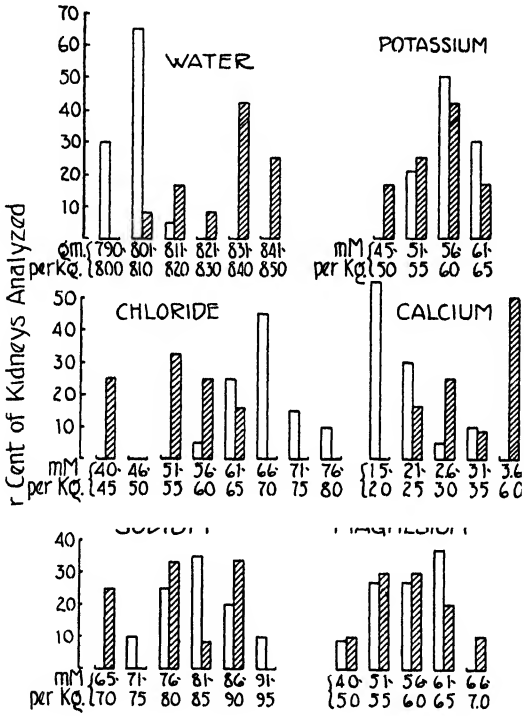

FIG. 1. The water and electrolyte values for normal and hydronephrotic kidneys are plotted against the percentage of kidneys analyzed to show the distribution. The ruled columns indicate the percentage of hydronephrotic kidneys; the clear columns, that of normal kidneys.

are distributed in different ratios in different portions of the kidney, so that an exact dissection cannot be made, it is always necessary to analyze the whole kidney if comparable data are to be obtained from different animals.

Hydronephrotic Kidneys—The analyses of hydronephrotic kidneys in Table III have been compared with the values for

normal kidneys in Table I by statistical (13) (Table IV) and graphic (Fig. 1) methods. It is apparent that the values for sodium, potassium, and magnesium have no significant differences. The increased water content of the hydronephrotic kidneys is highly significant, and agrees with the histological findings of dilated glomerular capsular spaces and fluid in the tubules. The decreased chloride content and the increased calcium concentrations are of doubtful statistical significance, but the probability of significant differences is somewhat increased by the distribution of values shown in Fig. 1.

Assuming that the difference in chloride concentrations is significant, this difference may be readily explained. Hydronephrotic kidneys are incapable of concentrating urine—therefore the presence of a urine concentrated in chloride in the tubules is eliminated. The lowest chloride concentrations, 42.2 and 41.2 mM, were found in the kidneys from Dogs To and J, respectively, whose chloride excretion was 6.7 and 6.0 mM per liter of urine. The increased concentration of calcium indicated in the hydronephrotic kidneys may be simply a reflection of the increased concentration of calcium in the serum of similar animals previously reported (1), or might possibly be the result of the presence of some infection in the kidney.

SUMMARY

1. Procedures are presented for water and electrolyte analyses on whole mammalian kidneys.

2. Total water, fat, and electrolyte concentrations were determined in kidneys from normal and from hydronephrotic dogs.

For normal whole kidneys the means were as follows: total water, 802.2 $\pm$ 5.6 gm.; chloride, 67.7 $\pm$ 5.3 mM; sodium, 82.6 $\pm$ 5.8 mM; potassium, 58.3 $\pm$ 4.0 mM; calcium, 2.16 $\pm$ 0.53 mM; and magnesium, 5.7 $\pm$ 0.5 mM per kilo of fat-free tissue.

The values for right and left kidney from the same animal were the same. The values for water, fat, sodium, and chloride were always more for the roughly dissected medulla than for the cortex. The significance of these increases was discussed. These data indicate the necessity of analyzing whole kidneys, since the distribution of water and electrolytes differs so distinctly in the medulla and cortex and since the two zones are distributed in

different ratios in different portions of the kidney, so that an exact dissection cannot be made.

The values for hydronephrotic kidneys, when compared statistically with those for normal kidneys, indicated that there was a significant increase in the total water content, but no change in sodium, potassium, or magnesium concentrations. However, there was a decrease in chloride and an increase in calcium, which were of doubtful statistical significance.

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THE REACTION BETWEEN IODOACETIC ACID AND DENATURED EGG ALBUMIN*

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(Received for publication, October 23, 1939)

The reaction between iodoacetic acid and sulfhydryl compounds has received much attention in recent years. Dickens (1) has shown that this reaction proceeds with the formation of HI and the substitution of a carboxymethyl group for the hydrogen of the thiol group. Smythe (2) has measured the rate of reaction of iodoacetic acid with various sulfhydryl compounds by estimation of the carbon dioxide change in a CO_2-bicarbonate buffer as a result of the HI produced. Rapkine (3) showed that iodoacetic acid may react not only with the sulfhydryl groups of relatively simple molecules, such as cysteine and glutathione, but also with those of proteins. It was found by Rapkine that as denatured egg albumin stood with increasing amounts of iodoacetic acid the intensity of its nitroprusside test diminished until it finally disappeared. Mirsky and Anson (4) have used this reaction in their method for determining available thiol groups in proteins. These investigators found that iodoacetic acid might react with only a portion or with none of the sulfhydryl groups of native proteins, as evidenced by the continued presence after the reaction of part or all of the original protein cysteine in the protein hydrolysate. Denaturation of the protein, however, causes all of the sulfhydryl groups to be capable of interaction with iodoacetic acid, there being, in this case, no free cysteine in the protein hydrolysate after the reaction.

In the experiments of Mirsky and Anson iodoacetic acid was allowed to react with coagulated denatured egg albumin; these

* This research was aided by a grant from the American Academy of Arts and Sciences.

authors state that 3 hours are required for the reaction to go to completion. In the present work, however, the denatured egg albumin was not permitted to coagulate. This was accomplished by adding 0.01 N KOH to a pure solution of egg albumin (prepared from the whites of fresh eggs by the method of Kekwick and Canaan (5)) to pH 7.3. The mixture was then placed for 10 minutes in water which had been brought to a boil. That iodoacetic acid reacts with this solution of denatured egg albumin much more rapidly than with a coagulum will be shown later. It is obvious that the interaction between iodoacetic acid and protein may be followed by measuring the iodide produced by the reaction. To this end a simple method for determination of iodides was devised.

The experiments were carried out in the following manner. To 3.5 cc. of egg albumin solution containing a known amount of the protein were added 1.5 cc. of 1 M phosphate buffer, pH 7.3, and 5 cc. of approximately 0.1 N iodoacetic acid which was previously neutralized with KOH. The reaction between the iodoacetate and protein was permitted to proceed for the desired period of time, at the end of which 0.25 cc. of concentrated H_2SO_4 and 0.25 cc. of 100 per cent trichloroacetic acid (10 gm. of trichloroacetic acid dissolved in water to make 10 cc. of solution) were added. The mixture was filtered and 0.1 cc. of 3 per cent H_2O_2 added to the filtrate. The depth of color of the iodine solution produced was then estimated, at the point of its maximum development, in a photometer, and the amount of iodide read from a previously prepared standardization curve. A blank was run with each experiment, which cancelled any iodide that was released by the iodoacetate itself.[1]

A typical experiment in which periods of reaction time from 1 minute to 6 hours were studied is shown in Fig. 1, Curve A. It may be seen that there is an initial rapid production of iodide lasting no longer than 10 minutes, after which the reaction slackens and after 40 minutes proceeds at a steady, much slower pace.

[1] A Sheard and Sanford photometer (Central Scientific Company) was used in these experiments. By this method quantities as low as 0.05 mg. of iodide in 10.5 cc. of solution could be estimated. In the region of 0.2 to 0.5 mg. of iodide, where most of the determinations fell, known amounts of iodide could be checked within 0.007 mg.

The initial sharp rise in iodide is without doubt mainly a result of the interaction between the iodoacetate and the thiol groups of the protein.[2] The cause of the continued slower rate of iodide production is, however, still a matter of conjecture. The work of Michaelis and Schubert (6) suggests the possibility that it results from the action of iodoacetate on amino groups of the denatured

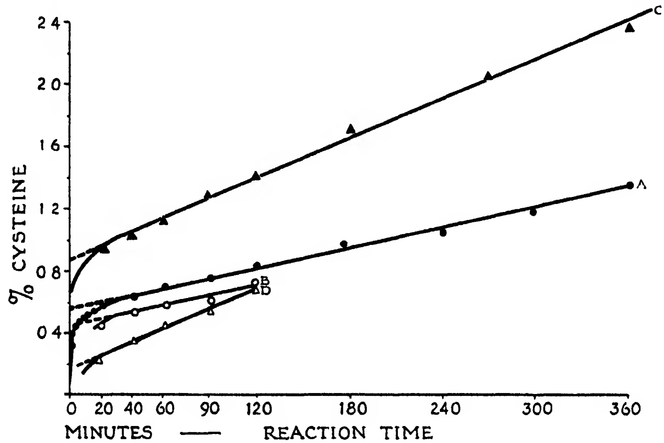

FIG. 1. The production of iodide upon reaction of iodoacetic acid with denatured egg albumin at pH 7.3. The iodide is expressed in terms of its equivalent per cent cysteine of the total weight of egg albumin.

$$\text{Mg. I}^- \times \frac{\text{mol. wt. cysteine}}{\text{mol. wt. I}} (= 0.95) \times \frac{100}{\text{mg. egg albumin}} = \%\ \text{cysteine}$$

Curve A, heat-denatured egg albumin which was treated with iodoacetate immediately following denaturation; Curve B, heat-denatured egg albumin which was treated with iodoacetate after standing 4 hours following denaturation; Curve C, urea-denatured egg albumin which was treated with iodoacetate after 1 hour in urea solution; Curve D, urea-denatured egg albumin which was treated with iodoacetate after 20 hours in urea solution.

egg albumin. However, cystine, tyrosine, arginine, and glutamic acid when subjected to the same experimental procedure yielded iodide at a rate much slower than would be required by this explanation. This observation, on the other hand, does not rule out the possibility of greater reactivity of the amino groups in the protein molecule under these conditions.

[2] The nitroprusside test was undiscernible after a reaction period of 5 minutes.

It is of interest to note that when a solution of native egg albumin was tested in the same manner as was the denatured protein no iodide was found at the end of a 5 hour period in the presence of iodoacetate. This indicates that not only the sulfhydryl but all of the groups in the denatured egg albumin which produce iodide with iodoacetate are non-reactive toward this substance in the native protein. Anson (7), however, has shown that they may not be inert toward all reagents.

It may also be observed from Fig. 1, Curve A, that groups other than sulfhydryl produce iodide as a straight line function with time. If this line for non-sulfhydryl iodide be extrapolated to zero time, a value for the iodide which results from reaction of the sulfhydryl groups is obtained. Assuming all of these sulfhydryl groups to be part of the cysteine moiety of the protein molecule, an index to the cysteine content of the protein is thus obtained. This treatment gives a value in heat-denatured egg albumin of 0.55 per cent available cysteine (average of ten determinations with a range of 0.53 to 0.57 per cent cysteine). This figure is in good agreement with those of Mirsky and Anson, 0.56 to 0.61 per cent (4), Todrick and Walker, 0.63 per cent (8), Kuhn and Desnuelle, 0.58 per cent (9), and Greenstein, 0.50 per cent (10).

The interesting observation of Greenstein (10) that denaturation of egg albumin by urea, guanidine, and various derivatives makes available a greater number of thiol groups than does heat denaturation made it appear worth while to study the reaction between iodoacetic acid and urea-denatured egg albumin. In these experiments 2 cc. of egg albumin solution (containing 30 to 90 mg. of egg albumin) were added to 2.4 gm. of urea, and the solution permitted to stand at 24–26° for 1 hour. (Greenstein has shown that the maximum number of —SH groups appears within half an hour.) Phosphate buffer and iodoacetate were then added and the determinations carried out as previously described. It may be noted from Fig. 1, Curve C, which shows data from a typical experiment, that the initial rapid rise in iodide (sulfhydryl) is markedly greater than that given by heat-denatured egg albumin. Extrapolation of non-sulfhydryl iodide to zero time yields a value of 0.87 per cent cysteine (average of eight determinations with a range of 0.82 to 0.91 per cent cysteine). This figure is lower than

that determined by Greenstein (about 1.00 per cent) by titration with porphyrindin dye. It is also of interest to note that the slope of the rate of production of non-sulfhydryl iodide is greater than that found with heat-denatured egg albumin. Thus, to the characteristics of urea denaturation as compared with heat denaturation may be added the observation that it causes a greater increase in availability not only of sulfhydryl groups but of other iodoacetate-reacting groups as well.

That the thiol groups of a solution of denatured egg albumin are quite labile is shown by the data presented in Fig. 1, Curves B and D. It was found that if heat-denatured egg albumin was permitted to stand for some hours (at pH 7.3) before being treated with iodoacetate the cysteine content calculated from the iodide released was less than that usually determined. That this disappearance of sulfhydryl groups does not represent a reversal of the denaturation of egg albumin (which has never been demonstrated) is evidenced by the fact that reheating of the solution, which would in this case redenature the egg albumin and give the original value for cysteine, caused no increase whatever in the sulfhydryl groups. This same phenomenon is also shown by urea-denatured egg albumin. On standing for 20 hours in urea the available sulfhydryl groups were markedly decreased. Since it is scarcely possible that denaturation could be reversed under these circumstances, this disappearance of the sulfhydryl groups probably indicates their oxidation.[3] On the other hand, the non-sulfhydryl groups which yield iodide with iodoacetate are apparently not thus susceptible to destruction, since their rate of iodide production is not diminished after standing.

SUMMARY

1. The reaction between iodoacetic acid and denatured egg albumin was studied by measurement of the iodide produced by the reaction.

2. In the course of this reaction there is an initial rapid production of iodide, which is believed to be caused by the sulfhydryl groups, followed by a slower steady yield of iodide due to some

[3] Hopkins (11) showed that the nitroprusside reaction of urea-denatured egg albumin eventually disappears on standing but that treatment with reducing agents causes it to return.

other group or groups as yet unidentified. Extrapolation of this non-sulfhydryl iodide to zero time yields a value for available cysteine of 0.55 per cent in heat-denatured and 0.87 per cent in urea-denatured egg albumin.

3. The sulfhydryl groups of the denatured egg albumin are labile, measurably diminishing in a few hours.

The author wishes to acknowledge with thanks the criticism and suggestions of Dr. H. B. Bull as well as the help of Dr. S. R. Gifford in the accomplishment of this work.

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AN ESTERASE FROM MUSCULAR TISSUE*

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The fact that liver contains an enzyme which is particularly adapted to the splitting of lower esters has long been known. Falk and Noyes (1, 2) have found a similar enzyme in the leg and uterine muscles of the rabbit. Otherwise the presence of an esterase in muscle appears to have been overlooked.

As reported in a previous paper (3), the authors found that benzyl butyrate is very rapidly hydrolyzed by lipase and esterase. It occurred to them that, since the products of hydrolysis are volatile with steam, the butyric acid produced could be separated and titrated without the interference of other substances. By this method they have shown the presence of a benzyl butyrate-hydrolyzing enzyme in the lean meat from various animals, including pork, mutton, beef, and fish. The principal study, however, has been made on beef muscle. Beef marrow had very little activity toward benzyl butyrate.

The presence of an ester-splitting enzyme in these tissues is definitely indicated by the behavior of benzyl butyrate. The better characterization of the enzyme, however, necessitated its separation, at least from the main bulk of the tissue. The first obstacle that arose was the fact that the enzyme is so firmly attached to the muscular protein that even autolysis does not liberate it, the liquid autolysate being practically inactive. Washed ground meat served equally well as a starting material and was used in the later experiments. Dilute sodium chloride,

* Food Research Division Contribution No. 469. This paper was originally presented before the Division of Biological Chemistry at the meeting of the American Chemical Society at Boston, September 11–15, 1939.

85 per cent glycerol, and digestion with chymotrypsin and with papain were tried without avail. It was finally found that active extracts could be prepared from well washed muscle by the use of dilute alkali, digitonin, digitalin, or saponin. Extracts made with digitonin or digitalin were clear liquids, while those obtained by the use of saponin and dilute alkali contained some finely divided protein in suspension. The general procedure for preparation of the active alkaline extract is shown in Fig. 1.

It seems evident from the methods required to bring the muscle esterase into solution that at least a considerable portion of the

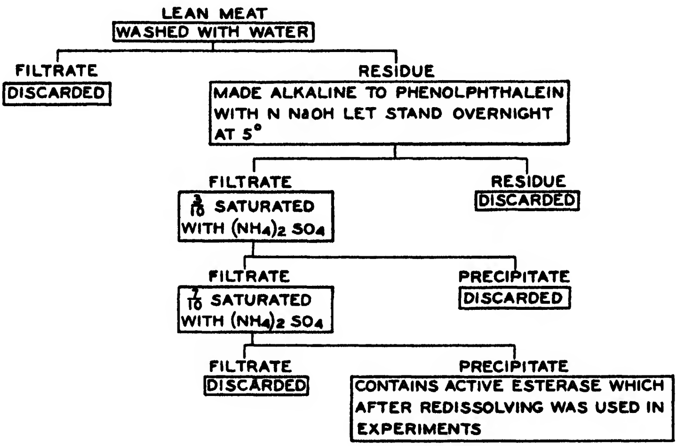

FIG. 1. Preparation of muscle esterase

enzyme exists attached to the muscle solids, rather than in the accompanying fluids. It therefore appears to be a true muscle enzyme.

A series of experiments on the alkaline extracts showed that the optimum hydrogen ion concentration was about pH 6. The best activity was obtained in the presence of phosphate. In contrast to liver esterase, hydrolysis at pH 8 was slow.

As indicated by tests on a series of substrates, the enzyme is an esterase similar to that in liver rather than to lipase, for only the glycerides and other esters of the lower fatty acids were hydrolyzed to any extent. Lecithinase, cholinesterase, and phosphatase were shown to be absent by tests on suitable substrates (Table I).

It was also found that the enzyme is considerably inhibited by bile, although it is active in the presence of digitalin, digitonin, and saponin, since active extracts could be obtained by the use of aqueous solutions of these substances. Synthetic experiments have been tried with these extracts but so far they have been

TABLE I

Activity of Esterase from Lean Meat

Substrate	Per cent digestion at 40°	
	5 hrs.	24 hrs.
Benzyl butyrate	49	76
Methyl propionate	3	26
" butyrate	34	66
" caproate	42	60
" laurate	0	1
" myristate	0	0
" oleate	1	2
" stearate	1	2
Ethyl propionate	5	11
" butyrate	14	53
" valerate	39	71
" caproate	22	44
" caprylate	5	12
" palmitate	0	0
" oleate	0	2
Glycerol tripropionate	8	31
" tributyrate	15	31
" trivalerate	4	8
" tricaproate	0	2
" trioleate	0	1
Lecithin	0	0
Acetylcholine	0	0
β-Sodium glycerophosphate	0	0

Butter 133 mg. per titration 2 hrs	0.05 cc. 0.1 N NaOH
5 "	0.08 " 0.1 " "
23 "	0.25 " 0.1 " "

rather inconclusive, owing possibly to the fact that the best physical conditions for the purpose have not been found.

The digestion mixture used to measure the activity contained 1.2 mM of substrate, 2 cc. of 0.5 M phosphate (pH 6), and 2 cc. of enzyme preparation in a total volume of 30 cc. Periodically,

5 cc. portions of the well emulsified mixture were dissolved in alcohol containing ether and titrated with alcoholic KOH, with use of phenolphthalein.

Owing to the present emphasis on the quick freezing of animal and vegetable food products, as well as on the older methods of low temperature preservation, it was thought desirable to investigate the action of this esterase at cold storage temperatures. Since benzyl butyrate had been found to be very easily hydro-

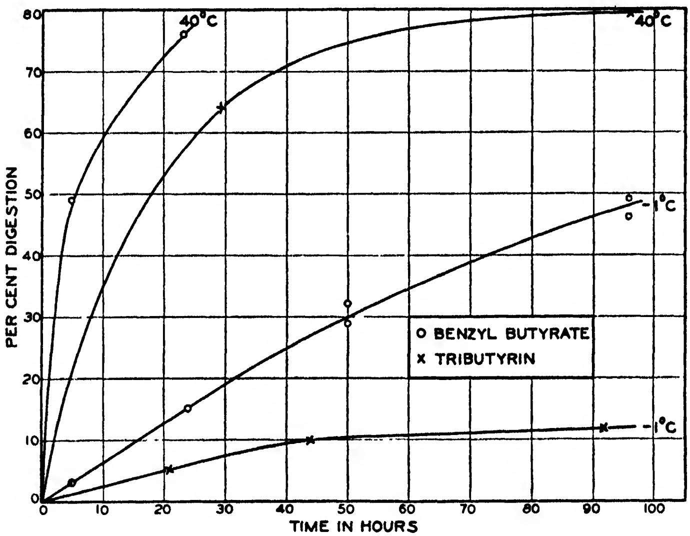

FIG. 2. Digestion of benzyl butyrate and tributyrin by meat esterase

lyzed, it was used for the first low temperature experiments with the enzyme extracted from the washed ground meat by the use of dilute alkali. After the fact was established that hydrolysis took place, tributyrin was tried under the same conditions. As can be seen from Fig. 2, the enzyme is quite active at freezing temperatures, such as would be used in cold storage.

A piece of lean meat was injected with benzyl butyrate and held at a temperature of $-7°$ for 4 days. 150 cc. of steam distillate required 17.6 cc. of 0.1 N sodium hydroxide solution for neutralization. A control held under the same conditions, the benzyl butyrate being added just before the distillation com-

menced, required 7.1 cc. of 0.1 N NaOH for neutralization. (A small amount of H_2SO_4 was added to hold back any volatile basic substances.)

To 4 gm. of thoroughly washed ground meat 0.5 cc. of benzyl butyrate was added and the mixture was held for 20 hours at $-1°$, 3 cc. of 0.5 N sulfuric acid were then added, and the whole was steam-distilled. The distillate was collected in 25 cc. portions until only 0.5 cc. of 0.1 N sodium hydroxide was required for neutralization of the liberated acids. The total 0.1 N alkali used was 3.6 cc. In an experiment at 40° and $3\frac{1}{2}$ hours 7.4 cc. of alkali were required for neutralization. The control required only 0.1 to 0.2 cc. for each 25 cc. of distillate.

In conclusion it can be stated that an enzyme exists in beef and other muscular tissues which has the characteristics of an esterase and which still shows marked activity at temperatures below the freezing point of water.

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STUDIES IN HISTOCHEMISTRY

XV. THE HISTOLOGICAL DISTRIBUTION OF CHOLINE ESTERASE IN THE ADRENAL GLAND

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Previous investigations concerning the quantitative histological distribution of biologically important constituents of the adrenal gland have been made for ascorbic acid (1) and lipase and esterase (methyl butyrase) (2). Since acetylcholine is evidently involved in the liberation of adrenalin, and because choline esterase is a factor which influences the fate of acetylcholine, it was decided to investigate the distribution of this enzyme in the adrenal gland. Furthermore, it was considered of interest to determine whether the general rule, that relatively high concentrations of acetylcholine in tissues are associated with relatively great choline esterase activities, applied to the different portions of the adrenal gland as well. Feldberg and Schild (3) found the acetylcholine concentration in the medulla to be 0.45 mg. per kilo compared to 0.1 in the cortex; hence, if the above rule holds, the medulla should have the greater enzyme activity.

Glaubach and Pick (4) were the first to show that the injection of a choline ester (urea chloroacetylcholine chloride, $NH_2CONH_2 \cdot ClCH_2COOCH_2CH_2N(Cl)(CH_3)_3$) resulted in an outpouring of adrenalin from the adrenals, and they suggested that choline esters might be intermediary agents in adrenalin discharge evoked by stimulation of the adrenal ganglion. Later Feldberg and coworkers (5), using acetylcholine, obtained results consistent with those of Glaubach and Pick, which they substantiated by subsequent experiments on the release of acetylcholine by splanchnic nerve stimulation (6). Additional evidence for the adrenalin secretory action of acetylcholine has resulted from the work of Broun and Beaune (7), Hermann *et al.* (8), and Chang and co-

workers (9), while Lévy and collaborators (10, 11) demonstrated that certain other esters of choline also possess this action.

EXPERIMENTAL

Beef, pig, and rabbit adrenals were used in this investigation. The glands removed directly after the killing were frozen with dry ice; cylinders of tissue were punched out, fixed to the head of a rotary freezing microtome, and slices 25 μ thick and 2.5 mm. in diameter were cut serially in the manner previously employed (1, 2). Two consecutive sections were taken for measurement of enzyme activity, the next slice for staining and histological examination, the following two for enzyme study again, and so on through the various layers of the gland.

The chemical micromethod of Glick (12), with the titration apparatus of Linderstrøm-Lang and Holter, was used for the determination of choline esterase activity and applied to measurements upon the microtome sections in the fashion employed earlier (13). Hydrolysis of the substrate, acetylcholine chloride, was allowed to proceed for 4 hours at 40°.

The sections removed for histological study were dipped in 95 per cent alcohol, transferred to water, and then stained with thionine and mounted in glycerol. These sections were used to identify the region of the gland. To enable correlation with the stained frozen sections and more detailed histological examination, the tissue surrounding the area from which the cylinder had been removed was fixed in 20 per cent formalin, embedded in paraffin, sectioned at 5 μ, and stained with hematoxylin and eosin.

DISCUSSION

Each experiment was performed on the adrenal from a separate animal, two of each species being employed. The results of typical experiments with pig, beef, and rabbit adrenals are given in Figs. 1 to 3. The enzyme activities were correlated with the cellular distribution by designations placed on the curves at those positions where the respective cells occurred in maximum concentration.

The gradual rise in the curve in Fig. 1, as the medullary region is approached, reflects the gradual increase in the proportion of medullary cells in the pig adrenal, while the more precipitous rise

in the curve in Fig. 2 results from the sharper transition of the reticularis to the medulla in the beef adrenal. The adrenal gland of the rabbit is intermediary in this respect. This is probably

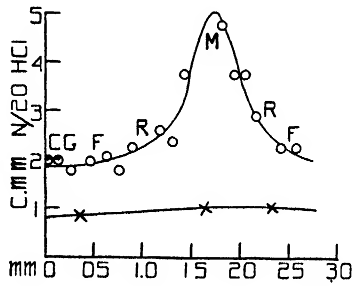

Fig. 1. Choline esterase activities in the various regions of the pig adrenal. ⊙ represents total hydrolysis expressed as acid formed in 4 hours at 40° in terms of 0.05 N HCl; × control values representing the sum of the non-enzymatic hydrolysis of substrate and titratable acid in the tissue. Histologically defined regions of the adrenal: *C* capsule, *G* glomerulosa, *F* fasciculata, *R* reticularis, *M* medulla. The position of the letters on the curve indicates the zone of the maximum concentration of the respective cells.

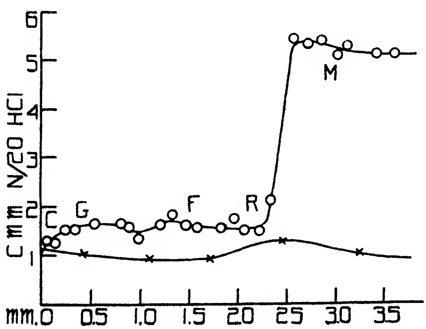

Fig. 2

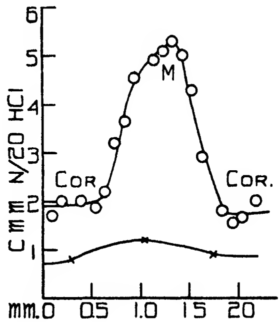

Fig. 3

Fig. 2. Choline esterase activities within the beef adrenal. The designations are the same as in Fig. 1.

Fig. 3. Choline esterase activities within the rabbit adrenal. The designations are the same as in Fig. 1. Cor. represents cortex.

due to the manner in which the tissue was removed with the punch, since the direction of the cylinder was not always strictly perpendicular to the medulla, so that in some sections both cortex

and medulla were present. The sharpness of the boundary between cortex and medulla in the individual glands may also be a factor which influences the gradient. In the rabbit, there is no clear histological differentiation between the fascicular and reticular layers of the cortex, and for this reason no designations of cell types were made on the curve other than indications of cortical and medullary regions.

There is little difference between the profiles of the curves for the three species studied, and it is obvious that the various cortical cells within a given gland possess a relatively low and constant enzyme activity as contrasted with the great activity of the medullary cells. In view of the fact that the choline ester responsible for adrenalin liberation must operate upon medullary cells, one might expect the greater choline esterase activity observed in this region of the gland. The ratio of 4.5:1 for the acetylcholine concentration in medulla and cortex found by Feldberg and Schild is approximately the same for the relative choline esterase activities. This parallelism is in agreement with the aforementioned general rule that high acetylcholine concentrations in tissues are associated with great choline esterase activities. It has already been suggested by Antopol, Glaubach, and Glick (14) that this association may be a control mechanism preventing the effects of excess acetylcholine in tissue. In the case of the adrenal, it is reasonable to assume that the choline esterase in the medulla plays a similar rôle.

SUMMARY

The quantitative histological distribution of choline esterase in pig, beef, and rabbit adrenals has been studied.

The cortical region of the adrenal possessed little enzyme activity, but the medulla was relatively potent.

No species differences were found, and no variations in the enzyme activities of the cell types in the cortex were observed.

The authors wish to thank Dr. Susi Glaubach for her valuable assistance in the preparation of the manuscript.

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PHOSPHATASE STUDIES: THE HYDROLYSIS OF AMINOETHYLPHOSPHATE AND β-GLYCEROPHOSPHATE BY FECAL AND KIDNEY PHOSPHATASE

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(Received for publication, November 20, 1939)

Outhouse (1) reported the isolation of a new phosphoric acid ester, aminoethylphosphate, from tumors. Recently Colowick and Cori (2) have isolated this same ester from the intestines of rats and rabbits. Although this ester is known to be hydrolyzed by both kidney and fecal phosphatase, no detailed study on the optimum hydrogen and magnesium ion concentrations has been reported. In order to fill this gap in our knowledge of this important ester, the investigation here reported was carried out. For comparative purposes the effect of the enzyme preparations on the commonly employed sodium β-glycerophosphate was also studied.

The fecal phosphatase used in the investigation was prepared by Armstrong (3) from dog feces at the Banting Institute and given to one of us (E.L.O.). Since that time it has been kept at room temperature in dark, stoppered bottles. The kidney phosphatase preparations were prepared from hog kidney by a slight modification of Armstrong's method for fecal phosphatase. Although it is by no means as active as Armstrong's fecal phosphatase preparation, nevertheless marked purification was accomplished. In its preparation 1300 gm. of hog kidney were ground in a meat grinder, suspended in 4000 cc. of 0.001 N NaOH plus 75 cc. of toluene, and allowed to autolyze at 20° for 5 days. The material was then placed in a refrigerator and filtered. The filtrate, which

* Presented by Russell V. Bowers in partial fulfilment of the requirements for the degree of Master of Science.

was quite brown, was cooled to about 2° and 80 cc. of 50 per cent acetic acid were added. After 10 minutes the pH of the solution was adjusted to approximately 9 with ammonium hydroxide and the solution filtered cold. The filtrate was saturated with ammonium sulfate and acetone added to give 60 per cent by volume. After the solution had stood for several hours, a brown precipitate separated out on top of the solution. This was filtered off and dissolved in 600 cc. of cold 1 per cent ammonium hydroxide. 6 gm. of wood charcoal were added, the solution stirred, and after some time filtered. An equal volume of acetone was added to the filtrate and the precipitate which formed was separated by centrifuging. The precipitate was washed successively with 60, 70, and 80 per cent acetone respectively. It was then placed in a vacuum desiccator to dry. The most active preparation under optimum hydrogen and magnesium ion concentrations, with sodium β-glycerophosphate as substrate, liberated 1.21 gm. of phosphorus per gm. per hour at 37.8°.

Determination of Optimum pH—The enzyme activity was determined by incubating solutions prepared in the following manner. To a Pyrex test-tube graduated at 15 cc. were added 5 cc. of a stock buffer solution, 5 cc. of a 0.01 M solution of the sodium salt of the ester being studied, and 1 cc. of a magnesium chloride solution of such a strength as to give the required magnesium concentration. The tube was then placed in a water bath at 37.8° and, after attaining the temperature of the bath, 1 cc. of the enzyme solution was added. The tube was shaken immediately and placed in the water bath for the required time. Controls without enzyme were run simultaneously. The phosphorus liberated was determined by King's method (4) at the end of the incubation period, the color being developed in the hydrolysis tube.

Several buffer mixtures were used in this investigation; namely, veronal-HCl, borate-HCl, and glycine-NaOH. The results obtained were the same for each of these buffer mixtures. The efficiency of these buffers under the conditions of hydrolysis was checked by determining the pH of similarly treated solutions both before and after incubation. The pH of the solutions was not significantly affected by the phosphorus liberated.

It will be seen from the experimental results which are graph-

ically represented in Fig. 1 that the optimum pH for fecal phosphatase on both sodium aminoethylphosphate and sodium β-glycerophosphate is 8.8. This value is considerably lower than the value of pH 9.6 reported by Armstrong (3) for fecal phospha-

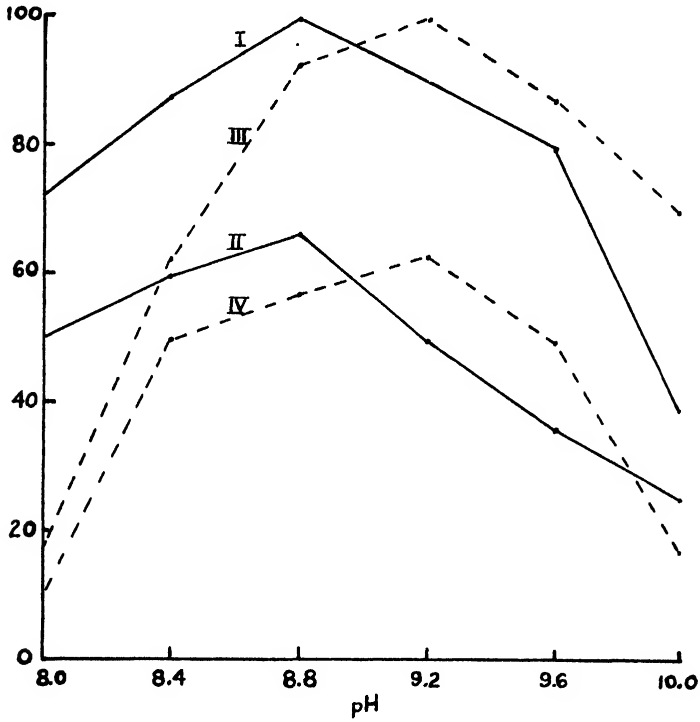

FIG. 1. The effect of pH on phosphatase hydrolysis. The ordinate gives relative hydrolysis with the amount of phosphorus liberated from β-glycerophosphate under optimum conditions taken as 100. Curve I, β-glycerophosphate-fecal phosphatase; Curve II, aminoethylphosphate-fecal phosphatase; Curve III, β-glycerophosphate-kidney phosphatase; Curve IV, aminoethylphosphate-kidney phosphatase.

tase acting on disodium phenylphosphate. The optimum pH for kidney phosphatase on both of the substrates employed was 9.2. This value agrees well with previous work on the action of kidney phosphatase on β-glycerophosphate (Kay (5), Albers and Albers (6)).

Determination of Optimum Magnesium Concentration—These experiments were carried out in the same way as the previous ones except that a borate buffer solution of a pH value correspond-

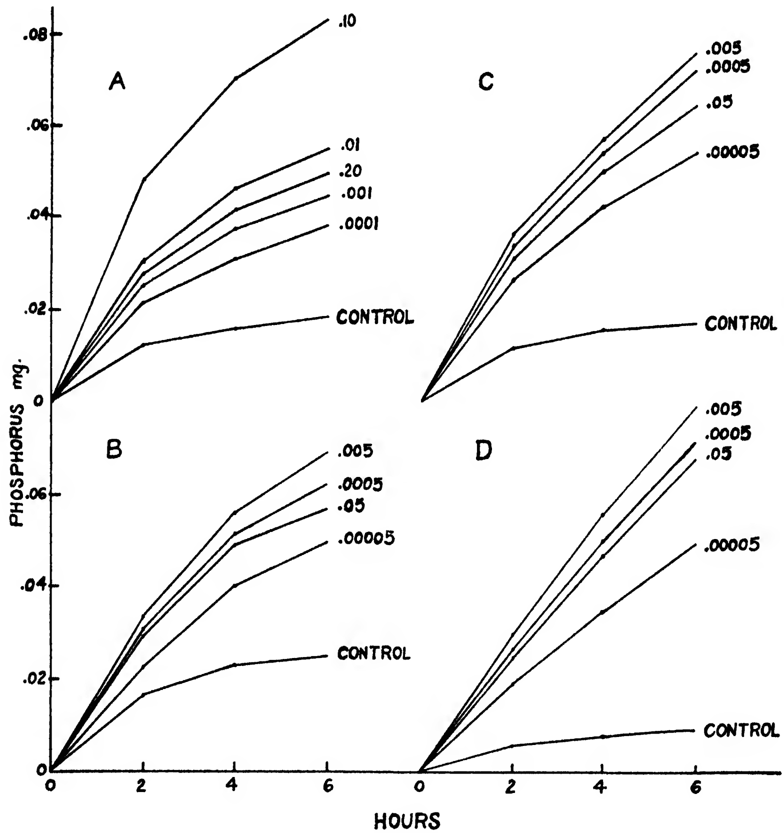

FIG. 2. The effect of magnesium on phosphatase activity. The magnesium concentration (in moles) is shown for each curve. *A*, aminoethylphosphate-fecal phosphatase; *B*, β-glycerophosphate-fecal phosphatase; *C*, aminoethylphosphate-kidney phosphatase; *D* β-glycerophosphate-kidney phosphatase.

ing to the optimum activity of the enzyme was used throughout; namely, pH 8.8 for fecal and 9.2 for kidney phosphatase. As a rule the incubation period was 1 hour, but in several instances, in order to get an activity curve over a longer period of time,

the experiments were run for 8 hours. Since these longer experiments gave results in complete agreement with the shorter one, for the sake of conserving space they alone will be reported. In these experiments of long duration a number of tubes of each magnesium concentration were incubated together and one of each analyzed for inorganic phosphorus at different intervals of time.

From the analytical results which are represented in Fig. 2, it will be seen that the optimum magnesium concentration for activation of fecal phosphatase, with sodium aminoethylphosphate as substrate, is about 0.1 M. A number of short duration experiments, not here reported, place the optimum as 0.08 to 0.1 M. When this enzyme acts on sodium β-glycerophosphate, the optimum magnesium concentration is markedly less, being only 0.005 M. The optimum magnesium concentration for kidney phosphatase, on the other hand, is the same for both substrates, namely 0.005 M.

The relative rate of hydrolysis of the two substrates by equal concentrations of enzymes, at different pH values and at optimum magnesium concentrations, is shown in Fig. 1. The results show that the rate of phosphorus liberation from aminoethylphosphate is only about two-thirds that from β-glycerophosphate.

DISCUSSION

The relative absence of magnesium in the enzyme preparations used in this study is demonstrated by the marked increase in activity on addition of magnesium. At optimum magnesium concentrations the activity is 2 to 3 times that of the enzyme without magnesium, an increase in activity comparable to that obtained by Jenner and Kay (7) with dialyzed phosphatase preparations on addition of magnesium.

The difference in the magnesium concentration required for optimum activity of fecal phosphatase when acting on aminoethylphosphate compared with that required when acting on β-glycerophosphate is of interest, the amount necessary for maximum activation, when aminoethylphosphate is the substrate, being about 20 times that required for β-glycerophosphate. This is a hitherto unknown characteristic of fecal phosphatase and, as far as we know, has never been reported for any phosphatase.

Since there is no apparent difference in the magnesium requirement of these two substrates when kidney phosphatase is used, this definitely indicates a fundamental difference between the two enzymes. The results suggest that the use of aminoethylphosphate as a substrate in the study of phosphatases might be of value in distinguishing between phosphatases from various sources.

SUMMARY

1. The optimum pH for fecal phosphatase acting on both sodium aminoethylphosphate and β-glycerophosphate has been found to be 8.8, while the optimum pH for kidney phosphatase acting on the same substrates is 9.2.

2. The optimum magnesium concentration for activation of fecal phosphatase is different for the two substrates, being 0.08 to 0.10 M for sodium aminoethylphosphate and 0.005 M for sodium β-glycerophosphate. Kidney phosphatase on the other hand required 0.005 M magnesium for both substrates.

3. The rate of hydrolysis of sodium aminoethylphosphate by both of these enzymes is only approximately two-thirds that of sodium β-glycerophosphate.

4. The possible significance of these variations of magnesium requirement for optimum activation with different substrates is briefly discussed from the standpoint of the phosphatase classification.

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A MICROMETHOD FOR THE DETERMINATION OF CARBON DIOXIDE IN BLOOD AND OTHER FLUIDS

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Christensen and Facer (1) have devised an apparatus and procedure for the microdetermination of carbon and hydrogen in organic compounds by a wet combustion process in which the evolved carbon dioxide is absorbed in standard barium hydroxide. The apparatus used for the determination is based on the principle of Pettenkopfer (*cf.* (2)) in which the carbon dioxide is drawn into an evacuated vessel containing the standard base. By slightly modifying the above apparatus of Christensen and Facer, it has been found possible to use it for the precise determination of the total carbon dioxide of plasma and other solutions.

The methods commonly used for such determinations are the manometric (3, 4), volumetric (5–7), and titrimetric (8) procedures of Van Slyke and associates. The manometric and volumetric methods, while precise, require rather expensive equipment, considerable experience in manipulation, and the use of certain factors peculiar to the solutions being analyzed. The titrimetric method involves the titration of a buffered solution, and, while being sufficiently accurate for clinical purposes, is not of research precision.

The procedure here presented has the advantages of requiring relatively simple apparatus and little manipulative skill, and yielding results comparable to those obtained by the manometric method.

Method

Apparatus—The apparatus, of Pyrex glass, is illustrated in Fig. 1. The reaction vessel *A* (volume, 10 ml. to the ground glass joint) was constructed from a 19/38 standard taper joint and a 2-way capillary stop-cock (*a*). The cup on the side arm contains about 5 ml. Flask *B*, for the absorption of CO_2, was made from a 250 ml. Erlenmeyer flask, a 29/42 standard taper joint, and a 2-way capillary stop-cock (*b*). Fig. 2 shows the pipette used for the protection and accurate measurement of the standard barium hydroxide solution. It was made from a 3 ml. Ostwald-Folin pipette.

Solutions—

1. Barium hydroxide. Approximately 0.05 N $Ba(OH)_2$ is prepared by adding 9 volumes of water to 1 volume of a saturated solution. When prepared, it is stored in a Pyrex flask protected by a soda lime tube and connected to the pipette described above.
2. Hydrochloric acid. Approximately 0.0300 N HCl was prepared and standardized against Na_2CO_3 purified according to Van Slyke and Neill (3).
3. Thymol blue indicator. 0.2 gm. of indicator is dissolved in 43 ml. of 0.01 N NaOH and diluted to 500 ml. with water.
4. Glycerol.
5. Caprylic alcohol.
6. H_2SO_4, 5 per cent by volume.

Procedure

The absorption vessel *B* (Fig. 1) is evacuated to approximately 30 mm. and filled with air drawn through a soda lime tower. It is then charged with 3 ml. of 0.05 N $Ba(OH)_2$ accurately measured with the pipette shown in Fig. 2, 3 drops of thymol blue indicator are added, and the flask is reevacuated. While the flask is being charged, care must be taken not to leave it open longer than necessary. It is very important that both ground glass joints be well lubricated with glycerol to prevent sticking. 3 to 5 drops of water are then placed in the U-tube connecting vessels *A* and *B*. 1 ml. of plasma, or other solution to be analyzed, is pipetted into the reaction vessel *A*, which is then connected (as shown in Fig. 1) to the absorption vessel *B*. 4 ml. of 5 per cent H_2SO_4 and

1 drop of caprylic alcohol are introduced into the cup on the side arm of the reaction vessel, which is then connected to a large soda lime tube.

Stop-cock *b* is carefully adjusted until a partial vacuum is attained in vessel *A*, as evidenced by the gases which pass through the trap in the U-tube. Stop-cock *a* is then opened and the acid in the side tube allowed to flow into vessel *A*. Stop-cock *a* is now partially closed and a slow stream of CO_2-free air permitted to enter, bringing flask *B* to atmospheric pressure in about 10 min-

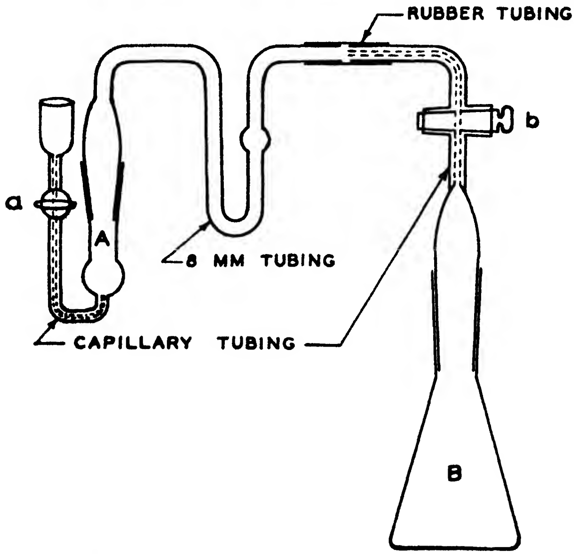

FIG. 1. Apparatus for carbon dioxide determination. *A*, reaction vessel; *B*, absorption flask; *a* and *b*, 2-way stop-cocks.

utes. After the air flow has been adjusted, flask *A* is placed in a water bath at 60–70° for the duration of the flushing.

After the system has returned to atmospheric pressure, stop-cock *b* is closed and the absorption flask disconnected and set aside for 15 minutes. During this time another determination may be started, for which a second absorption flask is used. 2 ml. of acetone are now added to the absorption flask and the excess $Ba(OH)_2$ is titrated with 0.0300 N HCl from a 5 ml. microburette. During titration the contents of the flask are protected from at-

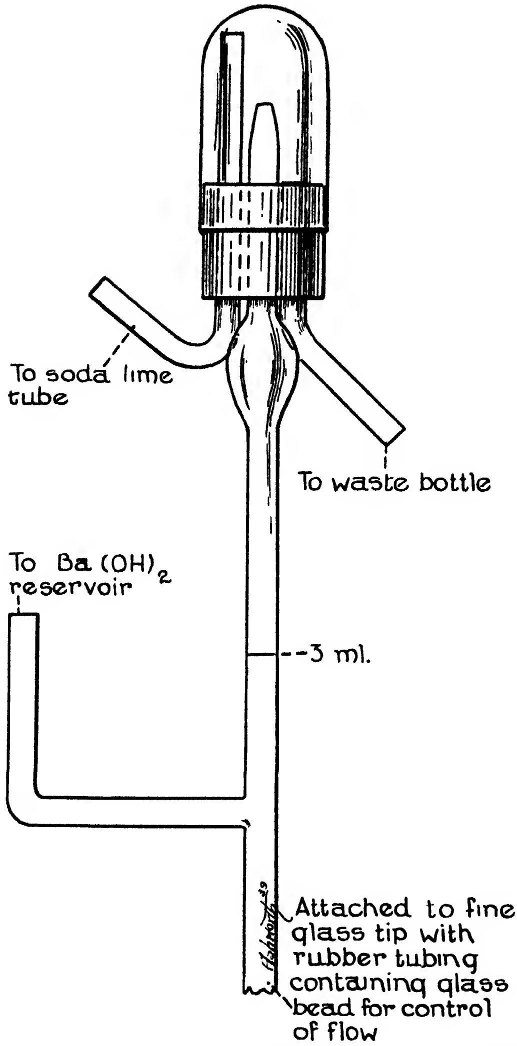

Fig. 2. Pipette for measurement of standard barium hydroxide solution

mospheric CO_2 by a rubber dam held over the mouth of the flask with a rubber band. The tip of the burette is admitted through a pin hole cut in the center of the cover.

Results

Blank Determinations—Since it is necessary to standardize the apparatus for small amounts of contaminating air, blank runs were made. 5.20 ml. of HCl were required to neutralize 3 ml. of $Ba(OH)_2$; in four blank runs 5.14, 5.14, 5.14, and 5.13 ml. of acid were required.

Analysis of Na_2CO_3 Solution—In order to determine the accuracy of the method, 1 ml. of a solution containing a known concentration of Na_2CO_3 was analyzed by this method and also

TABLE I

Determination of Plasma CO_2

Sample No.	Van Slyke	$Ba(OH)_2$ absorption
	vol. per cent	*vol. per cent*
1	40.9	41.1
	40.9	41.1
	40.6	41.4
2	36.4	36.6
	36.4	36.9
	36.6	36.3
3	59.4	59.5
	59.5	59.7
4	43.4	43.8
	43.4	44.1
5	48.4	48.5
	48.2	48.1
6*	56.7	56.5
	56.8	56.7

* This determination was made by a worker having no previous experience with the method, illustrating the fact that no great manipulative skill is necessary.

by the Van Slyke manometric procedure. The results of a number of typical runs are given (theory, 49.7 volumes per cent).

Van Slyke	$Ba(OH)_2$ absorption
vol. per cent	*vol. per cent*
49.4	49.9
50.2	50.6
49.4	49.9
50.1	49.7
50.1	

Analysis of Human Plasma—The total CO_2 content of human plasma was determined by the $Ba(OH)_2$ absorption method and the manometric method of Van Slyke. The results are given in Table I.

Calculations—The ml. of standard HCl required for the blank determination (A), minus the ml. of HCl required to titrate the excess $Ba(OH)_2$ (B), are equivalent to the CO_2 evolved. Expressed as an equation

$$\frac{(A - B)(\text{N of HCl})(44)}{2} = \text{mg. } CO_2 \text{ in sample}$$

and for a 1 ml. sample

$$\frac{(A - B)(\text{N of HCl})(22.4)(100)}{2} = \text{vol. \% } CO_2$$

SUMMARY

1. A simple inexpensive apparatus has been described for the determination of the total CO_2 content of plasma and other solutions.

2. It has been compared with the manometric method in carbonate solutions and plasma and found to agree within 1 per cent.

3. The time required for a complete determination is 15 minutes.

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PREPARATION, PROPERTIES, AND THIOCYANOGEN ABSORPTION OF TRIOLEIN AND TRILINOLEIN*

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The synthesis of glycerides of known structure has received the attention of many workers. Grün, in the recent book of Schönfeld and Hefter (1), has summarized much of the work which has been done in this field. However, most of the glycerides which have been prepared have been those of saturated acids. These glycerides are compounds which can be purified by crystallization, and the saturated acids from which they are made have been available in pure form. On the other hand, the liquid glycerides of the unsaturated acids have apparently not been prepared in a pure state and adequately characterized.

Several preparations of triolein have been reported. Berthelot (2) heated glycerol and oleic acid at 240° to obtain a product which remained liquid "to 10° and under." Carbon and hydrogen analyses agreed well with theory. Guth (3) prepared triolein by heating sodium oleate and tribromohydrin at 180° for 10 hours. The product solidified at $-4°$ to $-5°$. After several weeks, it solidified at ordinary temperatures; but after melting it again solidified at the lower temperature. The iodine number was 85.1 (theory 86.1) and the saponification equivalent was close to theory. The product distilled with decomposition at 235–240°

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Taken in part from material submitted by D. H. Wheeler to the University of Maryland in partial fulfilment of requirements for the degree of Doctor of Philosophy.

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at 18 mm. pressure. Pottevin (4) formed triolein by allowing an excess of oleic acid to react with monoolein at 36°, using 1 per cent of pancreatic tissue as catalyst. The separated triolein was said to solidify around 0° and to have a density of 0.915 at 15°. Bellucci (5) heated oleic acid and glycerol at 250° under 30 mm. pressure and purified the product by extraction with alcohol. His product had an iodine number of 85.7 and saponification equivalent close to theory.

Trilinolein was prepared by Izar (6) by heating potassium linoleate and trichlorohydrin at 160°. It was described as a reddish yellow oil. No further properties were reported. After completion of the present work, the announcement of the synthesis of mono- and trilinolein by Black and Overley[1] was noticed. They made the acid chloride of tetrabromostearic acid and from it prepared the triglyceride, which they debrominated to obtain trilinolein. Their product had an iodine number of 171.2 (theory 173.4), saponification number of 191.2 (theory 191.5), and n_D^{20} = 1.4709. They reported a melting point of −5° to −6° by the capillary tube method after the sample was held at −26° for several hours.

It is recognized that the glycerides of unsaturated acids play a very important rôle in relation to such properties of oils and fats as consistency, digestibility, nutritional value, ease of oxidation, and polymerization and other characteristics.

With a view of advancing the knowledge of the unsaturated glycerides and their properties, we synthesized triolein and trilinolein and obtained them in pure form. The method of preparation of these glycerides and some of their chemical and physical properties are described in this paper.

EXPERIMENTAL

Preparation of Triolein—The triglyceride of oleic acid was prepared by direct esterification of oleic acid and glycerol in an atmosphere of nitrogen with *p*-toluenesulfonic acid as catalyst.

The oleic acid contained not over 0.1 per cent of saturated acids or linoleic acid as impurities and was prepared as described in a previous publication (7).

[1] Presented before the Ninety-eighth meeting of the American Chemical Society at Boston, September 12, 1939 (Black, H. C., and Overley, C. A., *J. Am. Chem. Soc.*, 61, 3051 (1939)).

About 6 per cent excess of oleic acid was used to favor complete esterification of the hydroxyls. *p*-Toluenesulfonic acid was used in an amount equal to about 1 per cent of the combined weight of oleic acid and glycerol. The reactants were placed in the reaction vessel *E*, Fig. 1. The system from *C* to *I* was evacuated to about 1 mm., and then purified nitrogen was allowed to enter at *C* until a slight positive pressure was registered by the manometer *D*, after which a continuous stream of the gas was permitted to flow through the system. The nitrogen was purified by first passing over reduced copper (*B*) heated to dull redness by the

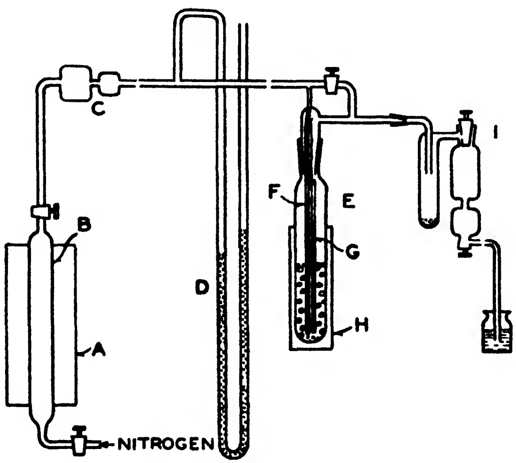

Fig. 1. Diagram of apparatus used for the preparation of triolein and trilinolein. *A* electric furnace, *B* reduced copper, *C* calcium chloride and anhydrous magnesium perchlorate, *D* manometer, *E* reaction vessel, *F* nitrogen tube, *G* thermometer, *H* electric heater, *I* absorption train.

electric furnace (*A*), and then over calcium chloride and anhydrous magnesium perchlorate (*C*). Heat was applied to the reaction vessel by means of a controlled electric heater, *H*. The temperature of the reactants was maintained at about 125°, as indicated by the thermometer, *G*. The stream of nitrogen led into the mixture by tube *F* served effectively to agitate the contents of the vessel and to carry over the water formed in the reaction to the absorption train (*I*) which contained calcium chloride and anhydrous magnesium perchlorate. The inert gas also prevented oxidation.

Water was evolved copiously at first; then gradually diminished as the reaction neared completion, until the amount of water evolved during ½ hour was less than 1 per cent of the total. Heating was then stopped, and the mixture was cooled and dissolved in 2 volumes of petroleum ether (b.p. 30–45°). This solution of the crude product was then washed with six or seven portions of 70 per cent ethanol. (A slight excess of potassium hydroxide was added in the first alcoholic washing to remove free acids.) After thorough washing with water, the solution was dried over anhydrous sodium sulfate, and the solvent was removed, finally, by warming under a vacuum (0.2 mm.) to constant weight. By this procedure 120.7 gm. of crude triolein were obtained from 131 gm. of oleic acid and 13.44 gm. of glycerol. In the reaction

TABLE I

Data Obtained from Molecular Distillation of Triolein

Fraction No.	Weight	Iodine No. (theory 86.1)
	gm.	
1	7.5	83.6
2	11.1	84.8
3	8.4	85.6
4	8.1	85.6
5	48.1	86.1
Residue.............	21.3	85.9

time of 5 hours 7.81 gm. of water (theory 7.88 gm.) were collected. The iodine number of the crude triolein was 85.3 (theory 86.1). The yield was 93.6 per cent, based on the glycerol.

Crystallization of the crude triolein from acetone (10 cc. per gm.) at $-45°$ gave 116.5 gm. of product having an iodine number of 85.5.

109 gm. of the crystallized product were then subjected to molecular distillation in a cyclic still like that described by Hickman (8). After the removal of traces of volatile material, the triolein distilled at a rate of 1 drop in 5 seconds, with a column temperature of 245° and at a pressure of 2 μ or less. The weights and iodine numbers of the fractions are shown in Table I.

The main portion, Fraction 5, had the theoretical iodine number for triolein. This fraction was used in further studies of the

physical and chemical properties of triolein. The saponification equivalent was 295.8 (theory 294.9); $n_D^{40} = 1.4621$; $n_D^{50} = 1.4586$; density at 40°, 0.8988. The molecular refraction calculated from the data at 40° was 270.5, compared to 269.0 for the sum of atomic refractions. Free acid, as oleic, was 0.05 per cent.

Melting and Freezing Points and Polymorphism of Triolein—From a study of melting and freezing points of triolein both by the capillary tube method and by cooling and warming curves, it was observed that polymorphism was exhibited similar to that reported by Clarkson and Malkin (9) in their work on saturated triglycerides, except that the transitions of the lower melting forms are more rapid.

From capillary tube observations, there appeared to be three crystalline or solid forms of triolein; Form I, the stable modification, melting at 4.7–5.0°, Form II, a form melting at about −12°, and Form III, a form melting around −32°. These observations were made as follows: The melting point tube containing triolein was placed in a bath at −55°, where the sample froze rapidly to a white solid. Upon slow warming of the bath, no melting was observed below 4.7–5.0°. However, if the sample frozen at −55° was immediately placed in a bath at −32° or higher, it melted to a turbid liquid and quickly solidified again (3 or 4 seconds). If the bath was below −32°, no melting occurred, even on warming the bath to −32°, or above, owing to the transition of Form III to II below the melting point of Form III. If the sample which had melted at −32° and resolidified was placed in a bath at −12° or higher, it again melted and quickly resolidified (4 or 5 seconds). Similarly, if the bath was below −12°, no melt was observed on warming the bath to −12° or above, until the melting point of Form I was reached, owing to transition of Form II to I.

The presence of the three forms was verified by cooling and warming curves. These observations were made with a calibrated potentiometer and thermocouple, permitting temperature determinations to an accuracy of 0.1–0.2°. The readings were obtained on a 5 cc. sample in a small test-tube, jacketed with a larger test-tube which served as lagging. The sample was stirred manually during the cooling.

The cooling and warming curves for Form I are shown in Fig. 2.

Cooling Curve A was obtained without seeding; Curve B showed less supercooling when the sample was seeded by a small amount of solid from a previous freezing. Warming Curve C agrees with the cooling curves in arrest point. From these curves, the freezing and melting point is 4.3°, a value only slightly lower than that obtained by the capillary tube method.

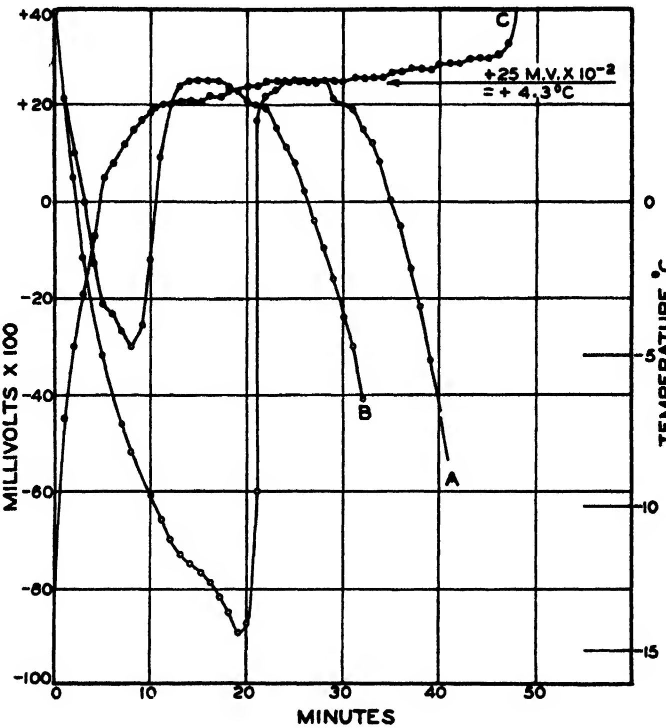

FIG. 2. Cooling and warming curves of triolein, Form I. Curve A, cooling curve, without seeding; Curve B, cooling curve, with seeding; Curve C, warming curve.

Form II could be demonstrated by a more rapid rate of cooling. Fig. 3 shows such a curve. The arrest for Form II is incomplete, and is followed by a rapid rise, almost to the melting point of Form I, due to heat of transformation of Form II to I. This partial arrest at $-8°$ was higher than the capillary melting point of Form II, probably owing to some change of Form II to I with

liberation of heat which displaced the incomplete hold due to the melting of Form II.

Evidence for Form III was difficult to obtain from cooling or warming curves, owing to its rapid transition. By very rapid freezing and subsequent warming at a rather rapid rate, an inflec-

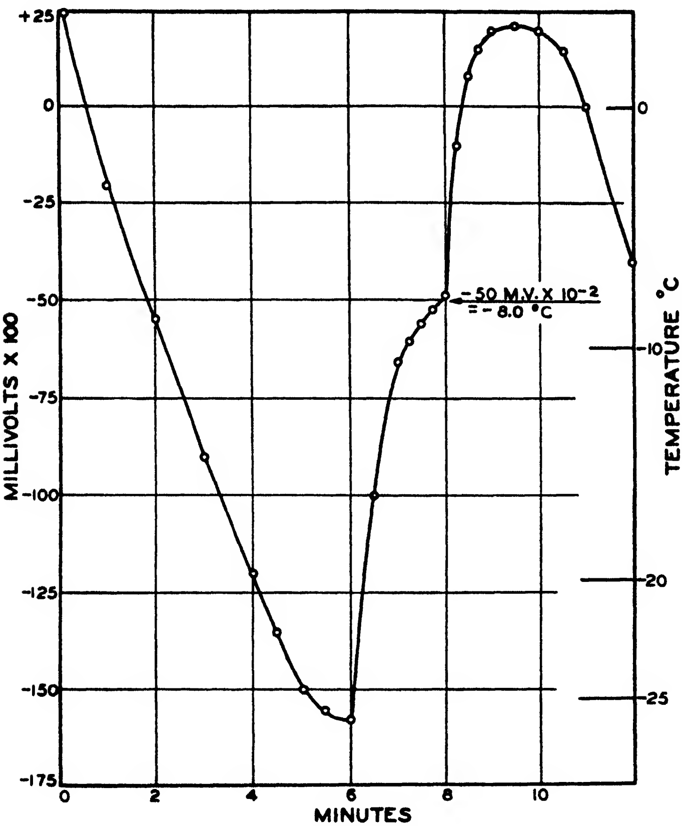

FIG. 3. Cooling curve of triolein, Form II

tion at $-24.6°$ was obtained (Fig. 4) which probably represents an incomplete hold due to Form III, influenced by the effects of transition to higher forms. (Only a very slight arrest for Form II, at about $-10°$, was also evident, followed by the rapid rise previously observed.)

Preparation of Trilinolein—Linoleic acid was prepared as previously described (10). The glyceride of this acid was synthesized in exactly the same manner as described for triolein. After 5 hours reaction time, 96.2 gm. of linoleic acid (iodine number 181.1), 10.25 gm. of glycerol, and 1 gm. of *p*-toluenesulfonic acid gave 5.77 gm. of water (theory 6.05 gm.) and 89.5 gm. of crude trilinolein (iodine number 171.5, theory 173.4). Yield, 93.3 per

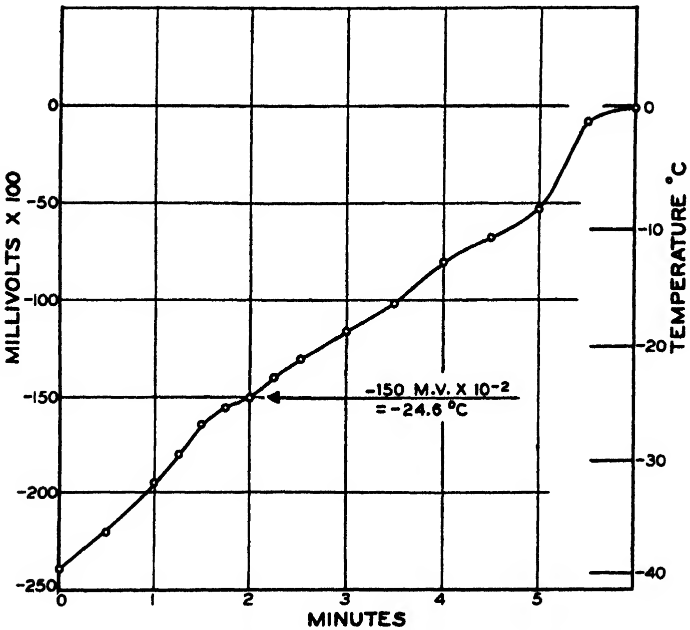

Fig. 4. Warming curve of triolein, Form III

cent, based on glycerol. Crystallization from acetone (10 cc. per gm.) at −65° afforded 82.2 gm. of product, iodine number 172.7.

The results of molecular distillation of 54 gm. of crystallized product prepared as above are shown in Table II. The trilinolein distilled at essentially the same rate as did triolein under the same conditions.

The trilinolein purified by molecular distillation was found to have the following constants: saponification equivalent, 292.4 (theory 292.9); free acid, as linoleic, 0.03 per cent, $n_D^{40} = 1.4719$ and $n_D^{50} = 1.4683$; density at 40°, 0.9184. The molecular refrac-

tion calculated from data at 40° was 267.9, compared to 267.4 for the sum of the atomic refractions.

Melting and Freezing Points and Polymorphism of Trilinolein—Only two solid forms of trilinolein were observed: By the capillary tube method Form I melted at from −13.1° to −12.8°; Form II melted at about −43°. Supercooling was more pronounced than with triolein. 15 to 20 minutes at −75° were required to freeze the sample. If the sample frozen in a capillary tube was placed in a bath at −43° or higher, it melted to a clear liquid and resolidified after 30 to 40 seconds. The product now melted normally at −13.1° to −12.8°. No intermediate forms were detected.

The cooling and warming curves for Form I are shown in Fig. 5. Supercooling was very pronounced without seeding (Curve A),

TABLE II

Data Obtained from Molecular Distillation of Trilinolein

Fraction No.	Weight	Iodine No. (theory 173.4)
	gm.	
1	5.1	169.5
2	7.8	172.6
3	6.6	173.8
4	11.2	173.9
Residue....	20.0	173.9

and the hold was shorter and 0.5° lower than that at −13.9°, the value obtained with seeding (Curve B). The warming curve (C) showed a melting point of −14.2°.

Evidence for Form II could be obtained only on warming curves of samples which had been frozen by direct immersion for 15 to 20 minutes in a bath at −75°. Fig. 6 shows such a curve with an incomplete arrest at −45.5°, followed by a rapid rise, due to heat of transformation of Form II to I.

Thiocyanogen Absorption of Triolein and Trilinolein—A previous study (11) of the thiocyanogen absorption of pure methyl oleate and methyl linoleate established a 3 hour reaction time as most accurate for mixtures containing relatively large amounts of the latter. It seemed desirable to extend this investigation to the

glycerides of these acids. The thiocyanogen numbers were determined by the modified Kaufmann method (12) at 20–23° for varying periods of time, as shown in Table III. Duplicate determinations agreed within 0.3 unit or less.

The values shown in Table III are averages of a number of determinations. It is evident from these data that a 4 hour reaction

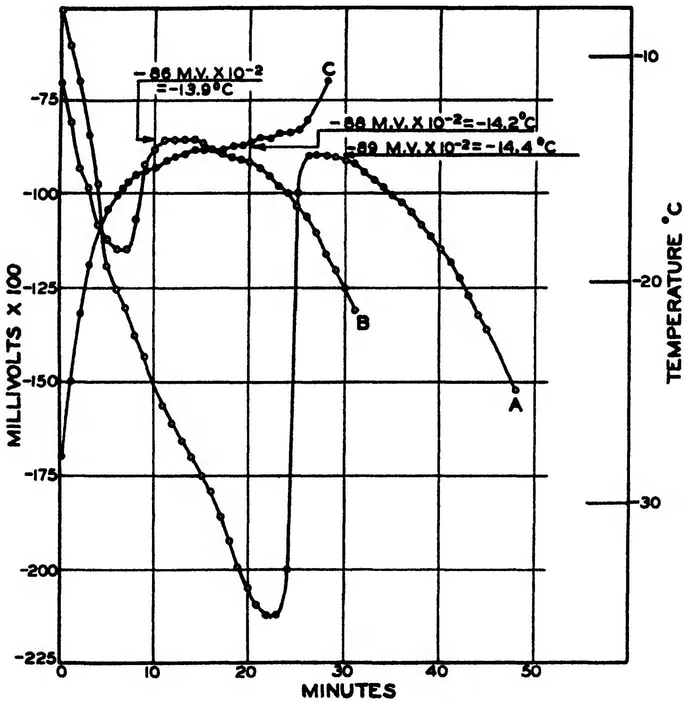

Fig. 5. Cooling and warming curves of trilinolein, Form I. Curve A, cooling curve, without seeding; Curve B, cooling curve, with seeding; Curve C, warming curve.

time is the best compromise, under the conditions employed, for determinations of the thiocyanogen numbers of triolein and trilinolein, especially in mixtures of both. The value at this period for triolein is slightly low (85.8, compared to 86.1 for theory) and slightly high for trilinolein (87.1, compared to 86.7 for theory). A shorter time gives values too low for both, while a longer time gives values too high for trilinolein.

Bromination of Trilinolein—It has been definitely established that bromination of linoleic acid produces about equal amounts of crystalline and liquid tetrabromostearic acids. Assuming a similar formation of equal amounts of solid and liquid bromine addition products from each of the linoleyl groups in trilinolein, it can be predicted that a 12.5 per cent yield of the triglyceride of the crystalline tetrabromostearic acid would be produced, along

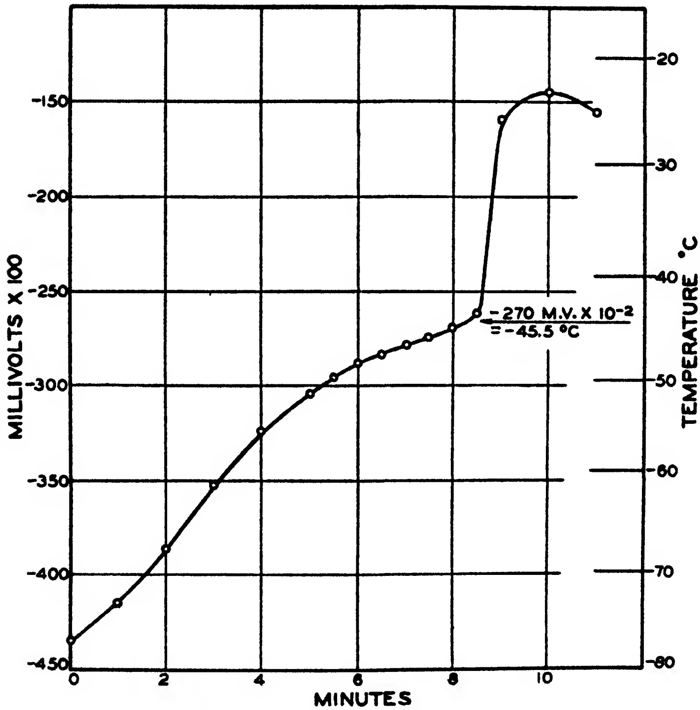

Fig. 6. Warming curve of trilinolein, Form II

with mixed glycerides of the solid and liquid bromo acids. The results of the following experiment support this prediction. Bromine in slight excess was added slowly with stirring to 5.01 gm. of trilinolein in 50 cc. of anhydrous ether cooled in an ice-salt bath. The excess of bromine was then removed with a few drops of amylene. The crude solid material, filtered from the cold reaction mixture, was recrystallized from ether at $-10°$ and then combined with the crystalline product obtained from the filtrates by the

addition of petroleum ether and cooling to about $-10°$. A 10.5 per cent yield, 1.095 gm., m.p. 73–78°, was thus obtained. Further crystallization of this material from petroleum ether and ether solution (2:1) afforded 0.707 gm., m.p. 81.0–81.7°, and 0.243 gm., m.p. 80–81°. The latter two fractions, 0.950 gm., represent a 9.1 per cent yield, which is about three-fourths of the 12.5 per cent yield predicted. The melting point is in good agreement with that reported by Black and Overley[1] for their synthetic triglyceride of crystalline tetrabromostearic acid (m.p. 81.0–81.5°). However, these investigators did not isolate a crystalline product upon bromination of their trilinolein in petroleum ether solution.

TABLE III

Thiocyanogen Numbers of Triolein and Trilinolein at Various Reaction Periods

Reaction period (20-23°)	Thiocyanogen Nos.	
	Triolein (theory 86.1)	Trilinolein (theory 86.7)
hrs.		
1	77.7	73.9
2		84.1
3	85.4	85.4
4	85.8	87.1
5	85.8	87.3
10	85.9	88.6
24	86.2	90.5

SUMMARY

1. Triolein and trilinolein have been prepared and purified, and certain of their physical and chemical properties have been reported.

2. The existence of three crystalline forms of triolein and of two crystalline forms of trilinolein has been demonstrated.

3. A study of thiocyanogen absorption has shown that a 4 hour reaction time is most suitable for the glycerides of oleic and linoleic acids.

4. Bromination of trilinolein produced a number of bromine addition products. A 9.1 per cent yield of crystalline bromides was obtained.

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COMPARATIVE STUDIES ON CREATINE, PHOSPHORUS, AND POTASSIUM IN VARIOUS MUSCLE TISSUES*

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Our present knowledge of the chemical behavior of creatine in muscle tissue rests largely upon the discovery of phosphocreatine by Fiske and Subbarow in 1927 (1). However, prior to this time there were many observations that suggested an interrelationship between creatine and other substances present in muscle. Urano (2) had already pointed out in 1906 the interesting fact that in experiments *in vitro* creatine and phosphate diffused from muscle at essentially the same rates, thus suggesting a connection between the two. In 1913, while working on the creatine content of rabbit muscle and its relation to urinary creatinine (3), it seemed probable to one of us (V. C. M.) that creatine was in some way linked with phosphorus, potassium, and glycogen in muscular activity, although in what way was not apparent. An attempt was made at that time to determine phosphorus and potassium as well as creatine in the muscle. The method for phosphorus did not prove entirely satisfactory, but a reliable method was available for potassium. A parallelism was noted between the muscle creatine and the potassium, but since no relationship seemed apparent at that time, the observations were not reported. However, in 1922, in writing a brief review (4) on muscle the conviction of a relationship between these substances was so strong that the statement was made, "Without further discussion it may be said that there are many observations which lead one to believe that

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glycogen, creatine, phosphoric acid and potassium are closely associated in active muscle."

The discovery of the acid character of phosphocreatine (5) has disclosed an important relationship between this substance and the acid-base balance of muscle tissue. Since it is a stronger acid than inorganic phosphate or hexosephosphate, its hydrolysis results in the liberation of base which becomes available to neutralize other organic acids formed during the contraction and recovery of muscle. The one base present in muscle cells in sufficient concentration to account for all of the base-binding power of phosphocreatine is potassium. The only alternative explanation apparent would be for the phosphocreatine to be bound to muscle proteins or to be present in some combined form not having the strongly acid properties of isolated phosphocreatine. In the absence of any evidence to substantiate the existence of such a combined form of phosphocreatine, it would seem more logical to postulate its existence as the dipotassium salt of creatinephosphoric acid.

The data presented in this paper include the potassium and creatine analyses carried out upon rabbit muscle in 1913 together with data upon the creatine, phosphorus, and potassium content of different muscles from man and a number of species of animals. The early creatine analyses are those previously reported (6). It has been our object to obtain figures upon muscles with widely varying concentrations of these three constituents in order to determine what relationship, if any, exists.

Methods

The earlier determinations of creatine were carried out upon an aqueous muscle extract, as described by Myers and Fine (3). Potassium determinations were carried out upon an aliquot of the same extracts as described by Myers (7) after ashing with sulfuric acid. Creatine, total phosphorus, and potassium determinations in the later studies were carried out as described in papers from this laboratory (8–10).

Results

Average values for the creatine, total phosphorus, and potassium of skeletal muscles from the hind legs of different animals

and for the pectoralis major muscle of man are presented in Table I. As will be seen, there is a close relationship among the three constituents. In general rabbit muscle has the highest concentration of each constituent, while human muscle has the lowest.

The data obtained in 1913 upon the creatine and potassium content of rabbit muscle after fasting and carbohydrate feeding are listed in Tables II and III. Since both fasting and carbohydrate feeding ultimately bring about a marked drop in creatine concentration, an excellent opportunity is afforded to compare any changes in potassium with those in creatine. As will be

TABLE I

Creatine, Phosphorus, and Potassium in Skeletal Muscle, Average Values

Animal	No. of individuals	Creatine	P	K	Creatine	P	K	Per cent K* (theoretical)
		mg. per 100 gm.	*mg. per 100 gm.*	*mg. per 100 gm.*	*mM per kg.*	*mM per kg.*	*mM per kg.*	
Guinea pigs	6	441	223	352	33.6	72.0	90.2	134
Humans, normal cases	11	443	201	328	33.7	64.7	84.0	125
Dogs	3	451	229	356	34.4	73.9	91.1	132
Rats	3	459	246	365	34.9	79.3	93.5	134
Rabbits	3	499	244	368	38.1	78.7	94.3	124

* Theoretical, calculated on basis of K required to account for all the creatine in terms of the dipotassium salt of phosphocreatine.

observed, there is a close correlation between creatine and potassium. It is of interest to note, however, that this is not a direct linear relationship. As the concentration of creatine becomes smaller, the ratio of creatine to potassium progressively decreases. This is due to a greater percentage decrease in creatine than in potassium. Such a shift argues strongly against tissue dilution as a major factor in the changes observed.

If one assumes that the changes occurring in these muscles are the result of chemical changes within the cells, a possible explanation of the observed changes may be made. The extreme lability of phosphocreatine suggests that it would tend to change more readily than the more stable compounds of muscle. Its

breakdown would liberate 1 mole each of creatine and phosphorus and approximately 2 moles of potassium. If these were not resynthesized into phosphocreatine or otherwise retained in indiffusible combination, one might expect them to diffuse out of the muscle. After a period of time a new equilibrium would be established. In each unit of the original tissue there would then be 1 mole less of creatine and phosphorus and 2 moles less of potassium.

If one averages the five cases in Tables II and III with the highest creatine content, average values of 44.1 and 107 mM of

TABLE II

Influence of Fasting on Creatine and Potassium Content of Rabbit Muscle, 1913 Experiments

Rabbit No.	Fasting	Creatine	Potassium	Creatine	Potassium	Δ creatine	Δ potassium
	days	*mg. per 100 gm.*	*mg. per 100 gm.*	*mM per kg.*	*mM per kg.*		
37	6	550	392	42.0	100.2		
41	7	573	473	43.7	120.8		
44	10	524	393	40.0	100.5		
24	11	380	364	29.0	93.2	15.1	14.2
40	12	417	291	31.8	74.5	12.3	32.9
38	15	492	347	37.6	88.8	6.5	18.6
43	17	426	382	32.6	97.8	11.5	9.6
42	19	357	333	27.3	85.3	16.8	22.1
36	22	402	334	30.7	85.5	13.4	21.9
39	24	361	258	27.6	66.0	16.5	41.4
16	27	370	284	28.2	72.7	15.9	34.7

creatine and phosphorus per kilo respectively are obtained. Using these values as a standard with which to compare the other cases with lower figures for creatine and potassium, one may calculate the relative decreases of the two substances as they are lost from the muscle. The figures in the last two columns of Tables II and III are the molecular differences between the creatine and potassium in each case and the mean of the five highest cases in the series. A similar series of values may be obtained if one used any other mean; for example, the lowest five cases or the mean of the entire group. It will be seen that the molecular decrease in creatine is accompanied by a greater

decrease in potassium which, in most of the cases, is about twice that of the creatine. If one takes the average creatine and potassium of the lowest five cases in the series, which average 27.4 and 76.7 mM of creatine and potassium, respectively, per kilo, the average difference between this group and the highest group is 16.7 and 30.7 mM of creatine and potassium. This represents a loss of 1.84 moles of potassium per mole of creatine.

If, on the other hand, one considers the possibility of simply removing part of the intracellular material, thereby increasing the relative amount of extracellular material (fat, connective tissue, and extracellular fluid), the result should then be a proportionate decrease in creatine and potassium. Since they are

TABLE III

Influence of Carbohydrate Feeding on Creatine and Potassium Content of Rabbit Muscle, 1913 Experiments

Rabbit No.	Carbohydrate feeding	Creatine	Potassium	Creatine	Potassium	Δ creatine	Δ potassium
	days	*mg. per 100 gm.*	*mg. per 100 gm.*	*mM per kg.*	*mM per kg.*		
61	4	643	399	49.1	102.0		
55	11	596	443	45.5	113.1		
60	10	480	367	36.6	94.0	7.5	13.4
54	19	373	299	28.5	76.5	15.6	30.9
53	24	367	355	28.0	90.9	16.1	16 5
57	29	482	349	36.8	89.4	7.3	18.0
46	53	339	269	25.9	68.8	18.2	38.6

present in the ratio of 1 mM of creatine to 2.5 mM of potassium, they should remain present in this ratio. Actually the ratio increases to nearly 1:3 in the lower cases.

To determine what relationship, if any, exists between the acid-soluble phosphorus and creatine, a number of different types of muscles from several species of animals have been studied. Average figures for the different muscles and brain are shown in Table IV. To bring out the relative manner in which the phosphorus shifts with respect to creatine, the average values for rabbit muscle have been taken as a point of reference and the differences between these values and those for the other tissues have been recorded. These values are designated in Table IV

as Δ creatine and Δ phosphorus. It will be seen that as the creatine content of the muscle decreases the total acid-soluble phosphorus also decreases, but at a slower percentage rate. This results in a decrease in the ratio of creatine to phosphorus. The molecular changes given in the last column of Table IV, however, show that there is actually a somewhat greater decrease in the number of moles of phosphorus than of creatine.

The values for creatine and phosphorus have been plotted against one another in Fig. 1. The solid line indicates the trend of the figures and the dash line has been drawn from the point

TABLE IV

Creatine and Total Acid-Soluble Phosphorus of Different Muscles and Nervous Tissue

Tissue	No. of cases or animals	Creatine	Acid-soluble P	Creatine to P ratio	Δ creatine	Δ phosphorus
		mg. per 100 gm.	*mg. per 100 gm.*	*mM*	*mM per kg.*	*mM per kg.*
Rabbit, voluntary muscle	10	572	200	0.68		
Human " "	8	430	177	0.58	10.8	7.5
Dog, voluntary muscle	3	382	134	0.68	14.4	21.4
" left ventricle	3	289	116	0.59	21.6	27.0
" right "	3	268	104	0.61	23.1	30.9
Rabbit, heart	10	244	113	0.51	25.0	28.0
Human left ventricle	8	220	120	0.43	26.8	25.8
" cerebellum	25	161	71	0.54	31.3	41.8
" right ventricle	8	156	85	0.44	31.7	37.1
" cerebrum	25	113	60	0.45	35.6	45.3
Dog, aorta	6	93	45	0.49	36.5	50.1

indicating the composition of rabbit muscle to the point of 0 creatine, assuming that 1 mole of phosphorus is associated with 1 mole of creatine. The dotted line through the origin represents the effect of dilution upon the system. With one exception (human voluntary muscle, which is only slightly outside) the points all lie within the area described by the dash and dotted lines. This is taken to indicate that in each case there are two factors operating: dilution of the intracellular phase and changes within the muscle cells themselves. Values lying close to the dotted line intersecting the origin differ from rabbit muscle chiefly as a result of differences in the amount of extracellular

material which makes up the bulk of the tissue. Those lying closer to the dash line, on the other hand, are interpreted as varying chiefly according to differences in the system concerned with phosphocreatine. We do not wish to infer that the changes in phosphorus and creatine represent an equal change in the phosphocreatine content of the tissues, but rather in the system directly concerned with the metabolism of this compound. We believe that the saturation level of creatine at any time must

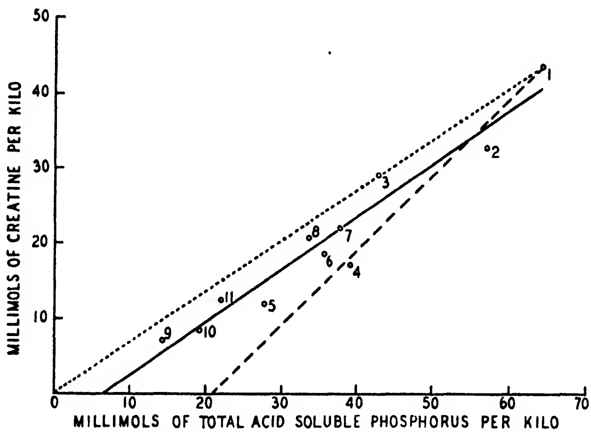

Fig. 1. The relationship of creatine to total acid-soluble phosphorus in different tissues. The solid line represents the average trend of values; the dotted line, the effect of dilution on rabbit muscle; and the dash line the effect of removing 1 mole of P for each mole of creatine. Curve 1, rabbit, voluntary muscle; Curve 2, human voluntary muscle; Curve 3, dog, voluntary muscle; Curve 4, human left ventricle; Curve 5, human right ventricle; Curve 6, rabbit, heart; Curve 7, dog, left ventricle; Curve 8, dog, right ventricle; Curve 9, dog, aorta; Curve 10, human cerebrum; Curve 11, human cerebellum.

depend upon the rate of the processes involved in its formation as compared with the rate of its breakdown. In the dog, the concentration of creatine is approximately 29 mM per kilo in skeletal muscle and 22 mM in the heart, a difference of 7 mM between the two. The concentration of phosphocreatine[1] is approximately 13 and 5 mM per kilo for cardiac and skeletal muscle respectively, a difference of 8 mM. From these figures

[1] Mangun, G. H., and Roberts, J. T., unpublished observations.

it would appear that the difference between the creatine and phosphocreatine of the two tissues is approximately the same. The acid-soluble phosphorus content of the two types of muscle differs by 5.6 mM in the three animals shown in Table IV.

DISCUSSION

The correlation between creatine and total acid-soluble phosphorus has been shown to be approximately the same for widely differing muscles from the rabbit, dog, and human. Creatine and potassium have also been shown to be closely related in voluntary muscle from the same species and different species. However, when one attempts to correlate the creatine and potassium of voluntary muscle with that of other types, no relationship seems apparent. While the creatine content of cardiac muscle is only half that of skeletal muscle, the potassium differs only to a small extent. However, it is quite possible that this discrepancy may be in some way associated with the higher phospholipid content of heart muscle. If one assumes that each mole of phospholipid combines with 1 mole of potassium, the extra phospholipid would account for approximately 20 mM of the potassium present in the heart. After deduction of this amount from the potassium of the heart, the relationship of the remainder to the creatine and total acid-soluble phosphorus is about the same as in voluntary muscle.

In other studies we have conducted upon the creatine, phosphorus, and potassium content of the human heart in myocardial insufficiency (11) we have found that slightly more than 1 mole of phosphorus and slightly more than 2 moles of potassium are lost from the heart for each mole of creatine lost. In more recent unpublished studies we have found that adenylpyrophosphate also breaks down in the dog heart in late aortic insufficiency, probably accounting for the excess phosphorus and potassium lost. In view of the parallelism in the behavior of these three muscle constituents in the heart and skeletal muscle under such widely different conditions, it hardly seems likely that this molecular relationship is accidental. The possibility that relative rates of diffusion played a significant rôle is minimized by the long duration of the experiments. The same is true of the human autopsy cases in which there is doubtless ample opportunity in

most cases for near equilibrium conditions before death intervenes. In late aortic insufficiency in the dog, for instance, there is no accumulation of inorganic phosphate as a result of the decomposition of phosphocreatine and adenylpyrophosphate. The total acid-soluble phosphorus and total phosphorus decrease, and this can be traced directly to an equivalent decrease in the phosphocreatine and adenylpyrophosphate.

SUMMARY

Creatine and potassium are shown to vary together in voluntary muscle from the same species and different species. As creatine decreases or increases, the change is associated with a variation in potassium in the approximate ratio of 1 mole of creatine to 2 moles of potassium, thus supporting the hypothesis that phosphocreatine is present in the form of the dipotassium salt.

A relationship is demonstrated between creatine and total acid-soluble phosphorus in different types of muscle and brain of a variety of species of mammals.

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THE UTILIZATION OF ACETONE BODIES

III. THE INFLUENCE OF ADRENALECTOMY

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That the adrenal gland plays a specific rôle in fat metabolism has been suggested for many years (1). More recently this hypothesis received further support when Long and Lukens (2) reported that adrenalectomy produces a marked reduction in the acetone body excretion of the depancreatized cat. This observation soon received ample confirmation by other investigators who found that adrenalectomy likewise reduced the excretion of acetone bodies, which follows phlorhizin intoxication (3), pregnancy (4), fasting (5), and the administration of extracts of the anterior pituitary gland (5, 6). The general conclusion derived from these observations implied that adrenalectomy inhibits the formation of acetone bodies and hence that the adrenal plays an essential rôle in fat catabolism.

The observation by MacKay and Barnes (5) that the adrenalectomized rat may develop a ketonemia after the administration of an extract of the anterior pituitary gland suggested to us that the adrenal glands may not be essential to ketogenesis. Consequently we studied simultaneously the blood and urine acetone body content of normal and adrenalectomized rats treated with an extract of the anterior pituitary gland. We found that whereas the acetone body excretion consequent to the administration of the extract was markedly decreased in the adrenalectomized rats, the blood acetone body level did not vary significantly from that of the pituitary-treated normal rats (7). From this it was con-

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cluded that adrenalectomy impairs the function of the kidneys in such a manner as to increase the threshold for the excretion of acetone bodies. The subsequent reports by Shipley and Long (8), Neufeld and Collip (9), and MacKay and Wick (10) support this conclusion and indicate that acetonuria *per se* cannot be accepted as a criterion of acetone body formation (ketogenesis) or acetone body utilization (ketolysis).

Although the studies mentioned above (8–10) indicate that the adrenals are not *essential* for ketogenesis, they do not eliminate the possibility that the adrenals may play some rôle in either or both ketogenesis and ketolysis. Therefore it became of interest to investigate this question further by some precise procedure.

In the present study we have concerned ourselves with the influence of adrenalectomy on acetone body utilization. In order to eliminate the possible influence of spontaneous ketogenesis, it was necessary to study conditions under which acetone body formation was at a minimum. This can be achieved by studying animals which have received an injection of glucose, for under such conditions endogenous ketogenesis is negligible (11, 12).

In order to obviate the effects of absorption, diffusion, and variable rates of excretion, we studied the disappearance of intravenously administered acetone bodies (β-hydroxybutyrate) from the whole bodies of fed, glucose-treated rats. The amount utilized by an animal was computed by determining the difference between the amount of acetone bodies injected and that found in the animal after a definite interval of time.

EXPERIMENTAL

This study was carried out with female rats from our stock colony which were kept on a stock diet[1] until a few minutes before the beginning of the experiment and which then received an intraperitoneal injection of glucose (100 mg. per 100 gm. of body weight). The animals were divided into two groups, in one of which the rats were operated upon 2 days previously under ether anesthesia and the adrenals removed and, in the other, only perirenal fat was removed. All animals after operation were given saline *ad libitum*.

[1] Purina Fox Chow.

Within 40 minutes after the administration of glucose, the rats were given an intravenous injection of synthetic sodium β-hydroxybutyrate and were immediately placed in individual 1 liter beakers. The dosage of the β-hydroxybutyrate was kept between 8 and 9 mM per kilo of body weight and was injected by means of a tuberculin syringe.

In order to correct for the initial content of acetone bodies analyses were made on groups of glucose-treated adrenalectomized rats and rats with the mock operation, receiving no β-hydroxybutyrate.

TABLE I

Effect of Adrenalectomy on Utilization of β-Hydroxybutyric Acid in Fed Female Rats

Corrected for initial content: adrenalectomized rats = 0.07 mM per kilo, rats with mock operation = 0.02 mM per kilo.

Experimental group (10 animals in each)	β-Hydroxybutyrate injected	Acetone bodies found 40 min. after injection	Acetone body utilization in experimental period	Reliability of difference*
	mM per kg.	*mM per kg.*	*mM per kg.*	
Mock operation	9.05	2.67	6.38 (±0.08)†	8.7
Adrenalectomy	8.33	3.35	4.98 (±0.137)	

* Reliability of difference = (difference of means)/(standard error of difference). Standard error of difference = $\sqrt{(\text{S.E.M.}_1)^2 + (\text{S.E.M.}_2)^2}$.

† Standard error of the mean, S.E.M. = $\sqrt{(\Sigma d^2/n)}/\sqrt{n}$.

At the completion of the experimental period of 40 minutes, the animals were killed by a blow on the back of the neck and the carcass was then ground up thoroughly in an ordinary meat grinder, the excreta in the beaker being thoroughly mixed with the ground tissue. Extracts of the ground rats plus excreta were prepared and their total acetone body content determined by the procedures outlined in previous reports (11, 12).

The pertinent data obtained by the above procedures are summarized in Table I. As in our previous studies (11, 12), only negligible amounts of acetone bodies were found in the glucose-treated rats, which indicates that spontaneous ketogenesis is at a minimum even in the adrenalectomized animals.

The utilization of acetone bodies is markedly depressed in the

adrenalectomized animals, as compared with the rats with the mock operation. Thus the rate of utilization of the adrenalectomized rats for the 40 minute interval is 1.40 mM per kilo of body weight slower than that of the group with the mock operation. This difference is statistically reliable, as indicated by the reliability of difference of 8.7.

DISCUSSION

In view of the preceding we can now state that following the administration of a ketogenic stimulus, the adrenalectomized rat, as compared to the normal, will show (*a*) a normal ketonemic response (7, 8, 10), (*b*) a diminished ketonuria (7–10), and (*c*) a diminished rate of acetone body utilization (Table I). The blood level of acetone bodies is dependent upon the difference between their rate of formation and their rate of excretion and destruction. Since the latter two are definitely diminished in the adrenalectomized animal, while the blood level is relatively unaffected, it becomes obvious that a similar decrease in the rate of acetone body formation must also occur. In other words, ketogenesis must definitely be diminished by adrenalectomy.

Of all the factors which have been studied with reference to their influence on acetone body utilization by the muscles, only one has been shown to be effective; *viz.*, the metabolic rate. Thus it has been demonstrated that the administration of thyroxine and the consequent increase in metabolic rate are associated with a definite increase in the rate of acetone body utilization (13), whereas neither insulin nor glucose is similarly effective. Hence the converse is not improbable and any mechanism which will reduce the metabolic rate will be associated with a reduction in the rate of acetone body utilization. If that be the case, it is probable that the removal of the adrenals results in a decreased acetone body utilization in virtue of the general decrease in metabolic activity that ensues (14). Likewise it is probable that the reduced metabolism associated with adrenalectomy may produce a decrease in the rate of ketogenesis in spite of the fact that the adrenals *per se* are not essential for acetone body formation.

SUMMARY

Adrenalectomy produces a marked reduction in the rate of acetone body utilization.

It is probable that a similar reduction in the rate of acetone body formation occurs.

It is suggested that these changes are due to a general decrease in metabolic activity consequent to adrenalectomy.

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THE ULTRAVIOLET ABSORPTION SPECTRUM OF PROLACTIN*

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Of the various hormones which have been suggested to be constituents of anterior pituitary gland extracts, prolactin is the first of these physiologically active substances to have been prepared in a highly purified form. The excellent method of Lyons (1) yields an amorphous product of high lactogenic potency, and other methods for the isolation of highly active prolactin preparations have been described (2). In a preliminary report, White, Catchpole, and Long (3) have reported the isolation of a crystalline protein with high lactogenic activity from highly purified, amorphous products prepared from beef pituitary glands according to the procedure described by Lyons. The constancy of the physiological activity after several recrystallizations and the data obtained from a study of the x-ray diffraction pattern led to the preliminary suggestion that the crystalline protein is identical with the lactogenic hormone of the anterior pituitary gland.

Although the crystallization procedure which has been described (3) has not repeatedly given crystalline preparations, and although the yields of the crystalline protein are unsatisfactorily small, a sufficient quantity of the material has been obtained to permit the initiation of experiments designed to characterize thoroughly the physical and chemical properties of prolactin.[1] Efforts are

* This investigation was aided by grants (to A. W.) from the Committee on Therapeutic Research, Council on Chemistry and Pharmacy, American Medical Association, and the Fluid Research Fund of Yale University School of Medicine.

[1] It may be mentioned here that the preliminary data reported for the elemental composition of crystalline prolactin (3, 4) have been found to

being made to develop a more satisfactory method of crystallization which will readily yield larger quantities of the crystalline hormone.

It is not necessary here to review the evidence which indicates that prolactin is a protein, inasmuch as this has been adequately considered (5). The acceptance of the view that the highly purified and the crystalline protein preparations are identical with the lactogenic hormone makes available the many tools of protein chemistry for the study of this hormone. The electrical mobility and homogeneity of crystalline prolactin, as measured in the Tiselius apparatus, have been reported (6). The present communication gives the data obtained in a study of the ultraviolet absorption spectrum of prolactin.

EXPERIMENTAL

Two prolactin preparations have been employed in this study, a crystalline product and a highly purified, amorphous preparation[2] made from beef pituitary glands by the method of Lyons (1). All the solutions were photographed at room temperature in aqueous medium at pH 7.4. The solutions were prepared by suspension of the prolactin in water, followed by the addition of an adequate amount of 0.1 N sodium hydroxide to effect solution. 0.1 N hydrochloric acid was then carefully added until the pH was adjusted to 7.4. The absorption curve was measured with the aid of a Spekker spectrophotometer and a small Hilger quartz spectrograph, with a tungsten steel spark as the light source. Measurements were also made with the continuous light of the hydrogen discharge tube in order to detect the presence of narrow bands.

Results

In Fig. 1 are plotted the absorption spectrum curves of crystalline prolactin and of a highly purified amorphous preparation.

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differ somewhat from subsequent analyses which have been conducted on other crystalline prolactin preparations. These data will be discussed in a future publication.

[2] The crystalline prolactin had a minimal effective dose of 0.06 γ when bioassayed by the 2 day "local" test method of Lyons and Page (7). With the same method of determining physiological potency, the highly purified, amorphous preparation was effective in dose levels of 0.1 γ.

It can be seen that the values of the absorption coefficients of the crystalline material are greater than those of the amorphous preparation. The positions of maxima and minima are about the same. The curve is typical of those found for a number of proteins, particularly those from animal tissue (8). The broad band with the maximum at about 2800 Å. is to be attributed to the combined absorption of the amino acids, tryptophane, tyrosine,

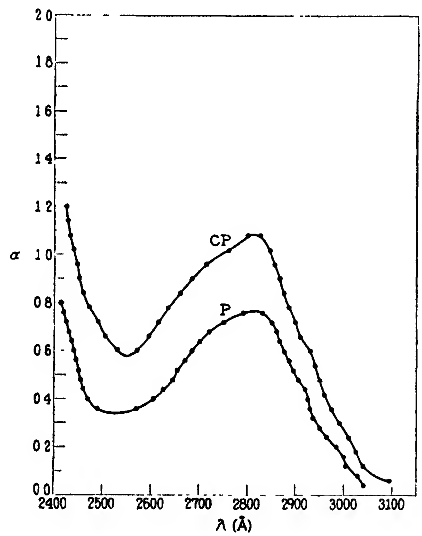

Fig. 1. Absorption curves of solutions of prolactin. Curve CP represents crystalline prolactin; Curve P was obtained with highly purified amorphous prolactin. pH 7.4. α = extinction coefficient, $\alpha = 1/cl \log I_0/I$, c = concentration (2.5 mg. per cc. in the case of Curve P, 1.6 mg. per cc. in the case of Curve CP), l = cell thickness in cm. (1 cm.), I_0 = initial intensity of light, I = intensity after passing through thickness l.

and phenylalanine, all of which have been demonstrated to be constituents of prolactin (5).[3]

When the absorption spectrum of prolactin is obtained with the continuous light source, a series of narrow bands is found. The bands have been read from the plates and plotted in Fig. 2. Although this latter method of illustration does not indicate the relative intensity of the bands, it can be used to indicate their

[3] White, A., and Bonsnes, R. W., unpublished data.

approximate position and to give some idea of their structure. For comparison purposes the narrow band spectrum of pepsin is also shown in Fig. 2.

The present interpretation of these bands is based upon results obtained from previous studies of the narrow band ultraviolet absorption spectra of other proteins (8, 9). The band at 2900 Å. is due to tryptophane, the one at about 2820 Å. is due to tyrosine, and those in the region of 2520 to 2680 Å. are due to phenylalanine.

In the case of prolactin, it can be seen that the phenylalanine bands are about the same as those found in pepsin and this also seems to be true for the tryptophane band; the absorption in the tyrosine region seems to be different from that found for pepsin in that the "tyrosine" band is apparently widened.

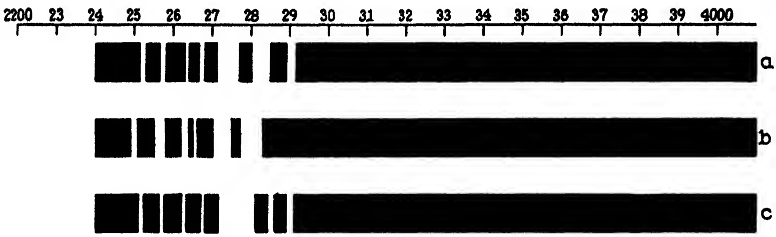

FIG. 2. Drawings of absorption spectra obtained with the continuous light source. *a*, crystalline prolactin; *b*, prolactin hydrolysate; *c*, pepsin.

As in the case of pepsin the tryptophane band is the most distinct when the plates are examined. The absorption in the tyrosine region is diffuse so as to make difficult the recognition of all of the band structure. The bands in the phenylalanine region are also quite diffuse. It was thought at first that these bands were not exactly like those given by pure phenylalanine in solution. However, owing to the diffuse nature of the bands, it is difficult to be certain of this at present.

In view of the diffuse absorption in the tyrosine region, it was desirable to obtain further evidence for the presence of tyrosine in prolactin. 50 mg. of crystalline prolactin were hydrolyzed by boiling under a reflux condenser for 18 hours with 20 per cent hydrochloric acid.[4] This hydrolysate following suitable dilution

[4] Suggested by Dr. J. S. Fruton, to whom grateful acknowledgment is made.

was photographed, the continuous light source being used. The bands obtained were similar to those given by a synthetic mixture of tyrosine and phenylalanine. A drawing of these bands is shown in Fig. 2, and it can be seen that, as would be expected, the tryptophane band has disappeared. The phenylalanine bands given by the hydrolysate appear to be more distinct than those found in the intact protein; in addition, there is a slight shift in position toward the shorter wave-length region of the spectrum.

SUMMARY

The ultraviolet absorption spectrum of prolactin has been measured and has been found to have a band with the maximum of absorption at about 2800 Å.

Photographs taken with the continuous light source of the hydrogen discharge tube show the presence of narrow bands. An interpretation of these bands has been proposed.

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THE STATE OF THE INORGANIC COMPONENTS OF HUMAN RED BLOOD CELLS*

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Under resting conditions—that is, when metabolism is reduced to a minimum by chilling—previous experiments have indicated that sodium, potassium, inorganic phosphorus, and phosphate esters will not traverse the membrane of the red blood cell even when the concentrations of water, sodium, potassium, or inorganic phosphate in the serum are greatly altered (1-3). However, when the metabolic processes in the red blood cells are activated by heating the cells to 37°, inorganic phosphorus may be made to pass in either direction across the cellular membranes (2, 3). Two explanations for the difference in the behavior of the active and resting cells present themselves. The permeability of the red blood cell membrane may vary with the metabolic processes. On the other hand it is possible that in the resting cell sodium, potassium, and phosphate are restrained by combination in undissociable or non-diffusible form with substances of such large molecular size that they cannot penetrate the membrane and that these are broken down in the reactions involved in the metabolic processes of the cells. In an effort to determine the relative importance of physical and chemical restraints in the segregation of the chemical components within the cell, the passage of sodium, potassium, and phosphate of hemolyzed blood across an artificial membrane was compared with their movements across the intact

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† This article represents work done in fulfilment of the thesis requirement for the degree of Doctor of Medicine at Yale University School of Medicine.

cell membrane. Such studies have been made before and have seemed to indicate that all the inorganic components of hemolyzed blood are freely diffusible and behave in accordance with the physicochemical laws that would govern the distribution of active ions between two fluid phases, one of which contained protein in high concentration. In these experiments, however, no attempt has been made to preserve the vitality of the blood.

The experiments to be reported may be divided into four groups. The first deals with the effect of hemolysis on the glucose and phosphorus of blood. The second treats of the ultrafiltration at 7° and at 37° of blood which has been hemolyzed by repeated freezing; the third of the ultrafiltration at 7° of blood hemolyzed by the addition of saponin. The last is concerned with the passage of sodium, potassium, and phosphorus across the membranes of intact red blood cells at 7° and 37°.

Methods

Venous blood drawn from non-fasting subjects was defibrinated by stirring with a glass rod and hemolyzed by repeated freezing or by addition of saponin.

Hemolysis by freezing was accomplished by lowering stoppered Pyrex tubes containing 4 or 5 cc. of blood into a thermos bottle which contained liquid air, where they were held until the blood was solidified. The tubes were then withdrawn and placed in water at room temperature until the blood had again become fluid. This process was repeated until hemolysis was sufficiently advanced. Later it was found that ethylene glycol monomethyl ether (commercially known as Cellosolve), cooled to about −78° by the addition of solid carbon dioxide, was a more convenient and efficient freezing medium than liquid air. The progress of hemolysis was followed by repeated blood counts. The red cell count dropped rapidly at first, but with diminishing rapidity in successive freezings (see Fig. 1). At least twenty freezings were required to reduce the count to 200,000 per c.mm. Since no bloods with original counts of less than 4 million cells were used, this means that at most only 5 per cent of the cells remained intact. This proportion of cells would contain a maximum of 0.5 mM of acid-soluble phosphorus and 2 mM of potassium (4, 5). The error that might be introduced by the restraint of such small

quantities of these substances did not warrant repeating the freezings further.

Hemolysis by Saponin—Since the quantity of saponin needed to hemolyze blood was not known, successive small amounts of the glucoside were added to blood until no intact cells could be detected under the microscope. It was found that complete hemolysis could be secured within 5 minutes by the addition to each cc. of blood of approximately 4 mg. of saponin.

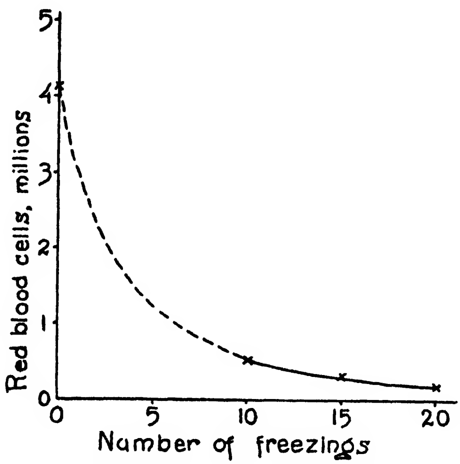

FIG. 1. The rate of destruction of red cells in blood hemolyzed by freezing.

Ultrafiltration—The hemolyzed blood was filtered under positive pressure through cellophane by the method described by Lavietes (6).

Controls—Samples of hemolyzed blood and of whole blood were allowed to stand beside each filter to serve as controls. At the end of the period these controls as well as the ultrafiltrate were subjected to chemical analysis.

Analytical Procedures

Analyses for potassium and sodium were made by the methods of Hald (7).

Both acid-soluble and inorganic phosphorus were determined by a micro modification of the method of Fiske and Subbarow (8).

1 cc. of blood or serum was introduced slowly, with constant agitation, into 6 cc. of 10 per cent trichloroacetic acid to which about 150 mg. of kaolin had been added. The mixture was then centrifuged. The kaolin was used to remove lipoid materials which sometimes cause filtrates of this concentration to become cloudy. The kaolin effectually eliminated this source of difficulty and, as careful controls demonstrated, did not interfere with the measurement of phosphorus. Ultrafiltrates were also diluted with 6 parts of trichloroacetic acid in order that the concentration of acid in all analytical mixtures might be approximately the same.

For the determination of inorganic phosphorus 0.5 cc. of distilled water, 0.2 cc. of 3 N H_2SO_4, and 0.2 cc. of 2.5 per cent aqueous ammonium molybdate were added to 1 cc. of protein-free supernatant liquid or diluted ultrafiltrate. Color was developed by the introduction of 0.1 cc. of aminonaphtholsulfonic acid solution. The standard was treated in the same manner except that 5 N H_2SO_4 was used instead of 3 N.

For the determination of acid-soluble phosphorus in whole blood 1 cc. of protein-free supernatant fluid was ashed with 1 cc. of 10 N H_2SO_4 and a few drops of concentrated HNO_3. The ash was then transferred quantitatively to a 10 cc. volumetric flask and diluted to the mark with water. In the case of serum and ultrafiltrate ashing was carried out with 0.55 cc. of 4 N H_2SO_4 and the ash was transferred to a 2.2 cc. volumetric flask. Each cc. of diluted ash, therefore, finally contained the same quantity of acid. To a 1 cc. aliquot of the diluted ash were added 0.7 cc. of water and 0.2 cc. of 2.5 per cent aqueous ammonium molybdate. Color was developed by the addition of 0.1 cc. of aminonaphtholsulfonic acid. The standard was treated just as was the standard for inorganic phosphorus. The error of this method does not exceed ± 3 per cent.

Glucose was determined by a micro modification of the method of Benedict (9) applied to Somogyi filtrates (10).

Results

Effect of Hemolysis on Glucose and Phosphorus Concentration of Whole Blood—Samples of hemolyzed blood were allowed to stand for several hours at 37° and 7°. As controls portions of the original blood were kept at the same temperatures. At varying

times all of these samples were analyzed for glucose and inorganic phosphorus.

Hemolysis, whether brought about by freezing or by treatment with saponin, tended to retard glycolysis at 37°. In one experi-

TABLE I

Glucose and Phosphorus Concentration of Blood, Hemolyzed by Freezing and with Saponin after Varying Time Intervals at 7° and 37°

All values are in mg. per cent.

Experiment No.	Temperature	Time	Control I*		Control II†		Hemolyzed with					
							Saponin at 4°		Saponin at 23°		Liquid air	
			Glucose	Inorganic P	Glucose	Inorganic P	Glucose	Inorganic P	Glucose	Inorganic P	Glucose	Inorganic P
	°C.	hrs.										
I	23	¾	78						82			
	37	2	68						78			
	37	17	0						0			
II	23	½		2.7						2.6		
	4	½				2.3		2.7				
	37	7	62		56		83		80			
	37	17	0	17.7	0		34	13.6	30	13.6		
III	23	0‡		2.5						2.6		2.7
	7	3	82	2.7					100	2.6		
	7	15	77	3.3					98	4.3		
	37	3	88	4.2					88	5.3	89	
	37	15	42	10.5					98	12.0	78	8.7
IV	23	0‡	52	3.5	65	3.2			59	3.8	59	3.7
	7	15	52	4.2	51	3.0			59	4.4	56	5.3
	37	3	23	5.3	23	4.8			68	8.0	45	10.7
	37	8	0	13.1	0	13.8			46	16.2	50	13.6
	37	20		20.4		20.3			0	18.8	0	17.3

* At 23° while blood was hemolyzed.

† At 4° while blood was hemolyzed.

‡ Immediately after hemolysis.

ment (Experiment III, Table I) the concentration of glucose in the hemolyzed blood remained unchanged. However, for the same reason glycolysis was not complete in the control blood, since glucose still remained after 15 hours at 37°. As was to be

expected, at 7° glycolysis was inhibited both in the hemolyzed blood and in the controls.

The inorganic phosphorus rose slightly, but definitely, immediately after hemolysis. It increased markedly in both hemolyzed and control bloods after standing at 37°. At 7° after 15 hours there was sometimes a small change in the controls, while in the hemolyzed samples there was a more definite and consistent rise.

In these experiments at least 2 hours were required to bring about hemolysis by freezing. Hemolysis with saponin consumed about half an hour, because repeated small quantities were added. Therefore, in order to determine the effect of varying time and temperature two control samples were set up. One sample of whole blood was kept at room temperature; the other stood in ice (Experiments II and IV, Table I). In one experiment (Experiment II) hemolysis with saponin was brought about at 23° and at 4°. All of these variations had little effect on the behavior of glucose and inorganic phosphorus.

Ultrafiltration of Blood Hemolyzed by Freezing—Freshly drawn blood was defibrinated and divided into two portions. One portion was kept as whole blood; the other was hemolyzed by freezing. As soon as hemolysis was complete, both portions were analyzed for inorganic phosphorus. In addition the whole blood was analyzed for acid-soluble phosphorus, potassium, and in some instances sodium. Samples of the hemolyzed blood were placed in two filters and allowed to stand at temperatures either of 7° or 37°. Tubes containing both whole blood and hemolyzed blood were placed beside the filters to serve as controls. After 18 hours the ultrafiltrate was removed and analyzed for inorganic and acid-soluble phosphorus, potassium, and sodium, while the controls were analyzed for inorganic phosphorus. The results of these analyses are presented in Table II.

Substrate was not analyzed directly, because its high viscosity made measurement almost impossible. It was assumed that hydrolysis of phosphate esters would be the same in the substrate as in the hemolyzed blood control. It was feared that lipoid phosphorus or protein phosphorus might possibly be liberated in the hemolyzed blood. However, three control experiments proved conclusively that total acid-soluble phosphorus remained unchanged under the conditions of these experiments. The

TABLE II

Ultrafiltration of Hemolyzed Blood

Experiment No.	Solute	Concentration per liter blood: Initial, Control	Concentration per liter blood: Initial, Hemolyzed	Concentration per liter blood: After 18 hrs., Control	Concentration per liter blood: After 18 hrs., Hemolyzed	Ultrafiltrate per 20 cc. blood	Concentration per kilo water: Substrate*	Concentration per kilo water: Ultrafiltrate	Concentration per kilo water: Ultrafiltrate/Substrate
Blood hemolyzed by freezing; filtered at 7°									
		mM	*mM*	*mM*	*mM*	*cc.*	*mM*	*mM*	
I	Inorganic P	1.08	1.38			2.5		2.46	
	Organic P	8.30						1.15	
	K	39.10					53.6	24.2	0.450
II	Inorganic P	1.14	1.16	1.17	2.20	3.0	2.83	2.36	0.833
	Organic P	16.89	16.69				23.90	0.46	0.019
	K	34.39					47.80	21.90	0.458
III	Inorganic P	0.75	0.88	0.75	2.21	3.0	2.96	2.10	0.710
	Organic P	12.68	11.70						
	K	40.44					52.4	42.34	0.808
	Na	77.50					98.0	91.30	0.932
IV	Inorganic P	0.71	1.36	0.74	2.62	3.0	3.40	2.72	0.801
	Organic P	8.37					9.52	1.23	0.129
	K		37.1				46.3	46.8	1.010
	Na		78.7				102.5	79.0	0.770
Blood hemolyzed by freezing; filtered at 37°									
V	Inorganic P	0.99	1.18	5.65	5.81	2.0	7.55	5.30	0.702
	Organic P	8.57					4.78	4.07	0.852
	K	36.7					47.5	34 5	0.726
	Na	81.4					100.0	116.7	1.167
VI	Inorganic P	0.83					7.17	5.33	0.743
	Organic P	11.26					8.17	8.24	1.007
VII	Inorganic P	0.75	1.66	6.50	6.50	2.0	8.44	5.87	0.696
	Organic P	7.68					6.00	6.43	1.070
	K	41.5					54.3	40.3	0.742
	Na	79.0					97.1	110.6	1.140
Blood hemolyzed by saponin; filtered at 7°									
VIII	Inorganic P	0.60	0.71	0.62	1.34	0.75	1.70	1.56	0.920
	Organic P	4.86					5.16	5.57	1.078
	K		33.56				44.2	7.84	0.177
	Na		91.20				114.5	122.5	1.070

TABLE II—*Concluded*

Experiment No.	Solute	Concentration per liter blood				Ultrafiltrate per 20 cc. blood	Concentration per kilo water		
		Initial		After 18 hrs.					
		Control	Hemolyzed	Control	Hemolyzed		Substrate*	Ultrafiltrate	Ultrafiltrate / Substrate
	Blood hemolyzed by saponin; filtered at 7°—*Concluded*								
		mM	*mM*	*mM*	*mM*	*cc.*	*mM*	*mM*	
IX	Inorganic P	0.70	0.92	1.35†	3.24†	1.25		1.97	
	Organic P	6.01							
	K	46.1					57.5	58.9	1.023
	Na	70.8					89.1	84.1	0.943
X	Inorganic P	0.83	0.98	0.75	2.76	1.0	3.46	3.08	0.890
	Organic P	5.98					4.41	3.36	0.763
	K	37.7					48.2	31.9	0.662
	Na	95.8					120.2	108.0	0.900

* These values in this column were calculated as described in the text; all others were directly measured.

† Blood stood 18 hours at 23°.

concentration of each constituent in the substrate was estimated by subtracting from the total amount of this substance originally in the blood the amount which had passed into the filtrate and dividing the residue by the volume of substrate that remained. These estimates are not exact, because measurements of volume could only be approximated. The ultrafilters, which were filled to capacity each time, were supposed to contain 20 cc. Actually some of them may have held as little as 17 cc. In every experiment as much ultrafiltrate as possible was removed for analysis. The total volume of ultrafiltrate was assumed to be equal to the sums of the volumes taken for analysis plus 0.5 cc. to account for the amounts lost in wetting pipettes and containers and left in the ultrafilter. Recovery tests indicate that these estimations may be too great by 0.2 cc.

To learn whether solutes are freely ultrafiltrable, it is necessary to compare concentrations per unit of water rather than concentrations per unit of volume. To estimate the proportions of water in substrate and ultrafiltrate, it was assumed that each sample

of blood originally contained 80 per cent and the ultrafiltrate 100 per cent of water.

The final concentrations of solutes in the water of substrate and ultrafiltrate and their distribution ratios are included in Table II. It is appreciated that quantitative deductions based on such rough measurements and so many assumptions must be made with caution. However, they do permit comparison of the relative filtrability of the various solutes at least.

Except for the initial burst immediately resulting from hemolysis inorganic phosphate remained unchanged in hemolyzed blood kept at 7°, but increased at the expense of organic phosphate esters at 37°. If it is assumed that hydrolysis proceeded at the same rate in substrate as it did in the control hemolyzed blood, the final concentration of inorganic phosphate was greater in substrate than filtrate. Since the Gibbs-Donnan equilibrium requires that the concentration in filtrate be higher and the cellophane membrane is quite permeable to the phosphate ion, this indicates that a certain proportion of the inorganic phosphate is restrained by some force other than the membrane or the Donnan equilibrium. The divergence from theory (more than 30 per cent) is so great that it can hardly be attributed to errors of analysis and measurement. Moreover it is quite as great in the 37° as in the 7° experiments, although in the former inorganic phosphorus concentrations are greatly increased.

At 7° the organic acid-soluble phosphorus of blood hemolyzed by freezing remains practically unchanged. Furthermore, although the esters of which this fraction of phosphorus is supposed to be composed are of relatively small molecular size and should be freely diffusible, only negligible quantities escaped with the ultrafiltrate. In this case the disparity of distribution is unequivocal. There cannot be a shadow of doubt that most, if not all, the phosphate esters are restrained, and it is hard to conceive of any restraining force of this magnitude other than combination with compounds of such large molecular size that they cannot traverse the membrane. When the blood is incubated, a large proportion of the organic phosphate is hydrolyzed. At the same time the remainder becomes ultrafiltrable, as if hydrolysis, under the influence of incubation, was accompanied by rupture of the combinations by which the organic phosphates were held at 7°.

The average distribution ratios of potassium, 0.682 and 0.734 at 7° and 37° respectively, are of the order of magnitude to be expected if the distribution were determined by the Gibbs-Donnan equilibrium in both sets of experiments. The variation of the ratios at 7° is, however, so great that it is somewhat dangerous to average them.

There can be no doubt, on the other hand, that sodium and potassium behave differently. In no instance are their distribution ratios the same. At 7° the concentration of sodium is higher in substrate than ultrafiltrate; at 37° the relation is, if anything, reversed. It is uncertain how much significance should be attached to this apparent departure from theory. It may be that this element, at 37°, distributes itself evenly throughout the water of both media without restraint. There can be no doubt, however, that it is not governed by the Gibbs-Donnan equilibrium and, therefore, probably is not combined with protein. Unlike potassium and organic phosphate, the major proportion of sodium in the filters was not derived from the cells, but from the serum of the blood. Not more than about 10 mM per liter of the sodium in each experiment originated in the cells. The total change of sodium in the incubator could, therefore, be accounted for if the cellular sodium were, like the cellular organic phosphate, completely unfiltrable at 7°, while the sodium of the serum was entirely free, and if incubation broke down the restraints by which cellular sodium was held, reducing it to the state of the serum sodium.

Until the experiments are reviewed by quantitatively more accurate methods, such explanations are merely speculative; but the differences in behavior of potassium and sodium belong in the realm of fact.

Ultrafiltration of Blood Hemolyzed with Saponin—The technique of the experiments in which blood was hemolyzed with saponin (Table II) followed closely the procedure described for the freezing experiments. Filtration was carried out only at 7°, because at 37° saponinized blood became so viscous that it would not yield enough filtrate for even a single analysis. Even at 7°, in order to obtain enough filtrate it was necessary to use four filters with approximately 10 cc. of hemolyzed blood in each.

In general the distribution of solutes resembles that of frozen blood ultrafiltered at 37°. It will be noted also that in saponin-

treated blood, although organic phosphate esters are preserved quite as completely as they are in frozen blood at 7°, they diffuse across the membrane as freely as they do in the frozen blood only at 37°. Sodium again is more evenly distributed than potassium, with ratios so near 1.0 as to give the impression that it filters without restraint. Again the distribution of potassium is extremely variable, averaging about what the Gibbs-Donnan equilibrium would predict. The variations seem altogether too large, however, to be attributed to analytical errors. Moreover, in every instance sodium was measured on filtrates of the samples from which potassium had been precipitated, and the sodium ratios are highly consistent.

Permeability of Red Cell Membrane to Sodium, Potassium, and Phosphorus at 7° and 37°—Freshly drawn whole blood, after defibrination, was divided into three portions. From the first, serum was separated at once and stored in the refrigerator. The other two portions of whole blood were allowed to stand for 18 hours at 7° and 37° respectively. Serum was then separated from both. These and the original serum were then analyzed for protein, potassium, sodium, and inorganic and acid-soluble phosphorus. The results of these analyses are presented in Table III.

The close agreement of the proteins at the beginning and end of each experiment shows that at 7° transfers of water were not appreciable. Both inorganic and organic acid-soluble phosphorus also remained unchanged. Potassium and sodium, however, seem to have crossed the cell membrane in opposite directions and in nearly equivalent amounts. The exchanges in Experiments III and IV are unmistakably large. While they are not so striking in Experiments I and II, they are nevertheless consistent in direction and equivalence. In previous experiments, referred to above (1, 2), these exchanges were not discovered for certain reasons. First of all, the blood was allowed to stand only until equilibrium had been attained, not 18 hours as it was in the present experiments. Secondly it was analyzed only for total base or for sodium. The exchanges here presented would, of course, have escaped detection by total base analyses because the transfers of potassium and sodium balance one another. In most instances, moreover, they were so small that one would have hesitated to attribute any significance to them from measurements

of sodium alone, because the differences do not greatly exceed the error of the sodium method. On the other hand the changes of potassium are far beyond the limits of technical error. Obviously these exchanges and other chemical reactions that may accompany them require further examination.

At 37°, judging from the regular increases of serum protein, water in every case entered the cells. In order to discover whether other solutes crossed the cell membrane, therefore, it is necessary to estimate what their concentrations in serum would have become

TABLE III

Diffusibility of Sodium, Potassium, and Phosphorus across Red Cell Membrane after 18 Hours Standing at 7° and 37°

Experiment No.	Serum protein	Sodium		Potassium		Phosphorus	
		Observed	Calculated	Observed	Calculated	Inorganic	Organic
	gm. per cent	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
I. Control	5.98	134.4		3.14		1.03	0.07
7°	5.98	132.8	134.4	4.41	3.14	1.01	0.07
37°	6.44	141.0	144.0	3.88	3.38	5.65	0.32
II. Control	7.26	138.0		4.44		1.05	0.35
7°	7.38	139.8	140.2	5.00	4.51	1.02	0.39
37°	8.10	142.5	154.0	4.76	4.95	6.59	0.16
III. Control	7.51	140.8		4.03		1.05	0.04
7°	7.61	137.7	142.5	10.02	4.08	1.05	0.04
37°	8.35	148.3	156.0	6.39	4.48	6.97	0.34
IV. Control	6.12	135.9		4.10		1.38	0.01
7°	6.43	139.0	142.6	8.68	4.30	1.38	0.01
37°	7.07	146.1	157.0	5.58	4.74	7.35	0.31

as a result of the water exchange alone by the equation $(\text{protein}_2)/(\text{protein}_1) \times S_1 =$ calculated S_2, when the subscripts 1 and 2 represent the sera at the beginning and end of the experiment respectively and S the solute in question. These calculated values are given in the right-hand columns under each solute in Table III. The differences between these and the observed concentrations indicate the quantities of solute transferred.

Inorganic phosphate rose so much that the increment can only have been derived from the organic esters in the cells. On the other hand, the organic acid-soluble phosphorus of the serum did

not rise. Analyses of blood showed that after 18 hours at 37° from 2.2 to 4.0 mM of organic acid-soluble phosphorus per liter still remained in the cells. The esters of which this was composed must, therefore, have been unable to traverse the cell membrane. While changes in potassium were relatively small, they were too great to be referred entirely to transfers of water. Some potassium seems to have escaped from the cells in every case. On the other hand, since sodium rose far less than protein, it must have passed into the cells. The quantities of sodium that left the serum, moreover, were far greater than the potassium that entered. For phosphate and sodium to move in opposite directions may seem anomalous at first, but it must be recognized that the blood becomes acid in the incubator.

DISCUSSION

The sudden increase of inorganic phosphorus immediately after blood is hemolyzed indicates that the integrity of the cellular contents is not entirely preserved. Further evidence to this effect is found in the retardation of glycolysis. That vital processes are not entirely abolished is evidenced by the fact that glycolysis does occur, though at a reduced rate. It is rather generally stated that rupture of the cellular membranes abolishes glycolysis. This statement rests upon experiments in which blood was hemolyzed by the addition of water (11, 12). Obviously the method by which hemolysis is effected is not a matter of indifference. Frozen blood differs strikingly from blood to which saponin is added. The former, in consistency, more clearly resembles intact blood; the latter is far more viscous. These differences are reflected in chemical behavior. Frozen blood acts much as does intact blood so far as phosphates are concerned. The organic esters remain intact as long as the blood is kept cold, but break down at incubator temperature. For this reason and because freezing has proved to be the best means of preserving the chemical composition of muscle cells, it seems reasonable to suppose that the state of the cellular constituents of blood will be least altered by this procedure.

If this assumption is justified, it may be inferred that organic phosphate esters in the intact resting cell are restrained from escaping by some force in addition to the membrane of the cell. The simplest restraining force that can be conceived is chemical

aggregation or combination with substances of large molecular size. At incubator temperature under the conditions of these experiments, without provision of nourishment or oxygen, the chemical reactions in the blood are not to be regarded as normal metabolic activities, but rather as processes of degradation or disintegration. In intact blood they can be reversed by addition of glucose (3); whether such a reversal could be effected after hemolysis remains to be determined. If the reactions in hemolyzed and intact blood are the same, these degradation processes involve not only the breakdown of a certain amount of organic phosphate but also resolution of the chemical restraint placed upon the remainder of the phosphate esters.

If the reactions in hemolyzed and intact blood are the same, it becomes at once apparent at this point that the effect or purpose of the chemical restraint is not to prevent loss of organic phosphate from the cell. This is effectively prevented by the physical restraint imposed by the membrane. At 37° in hemolyzed blood the organic phosphate residue moves freely across cellophane, but in intact blood no appreciable amount escapes from the cells. If the cellular envelope is impervious to free organic phosphate esters, as these experiments suggest, the chemical restraint must serve only to diminish or abolish the osmotic activity of the phosphate esters. The cells would by this means be enabled to vary their load of these important constituents without being compelled to assume or deliver an equivalent amount of water. The variability of the concentration of organic phosphate so far exceeds that of water or other chemical constituents of the red blood cells that some such osmotic defense would have to be postulated, even if it could not be demonstrated.[1]

The saponin experiments served two purposes: first to demonstrate that the chemical restraint can be dissociated from the reactions that attend glycolysis; second to prove that the non-filtrability of organic phosphate of frozen blood at 7° was not referable merely to the temperature. Cold inhibited glycolysis and hydrolysis of phosphate esters quite as effectively in the blood

[1] In the present experiments organic phosphorus of whole blood varied from 4.8 to 16.7 mM per liter. In blood with the normal proportions of cells containing the usual amounts of water, this would be equivalent to 15 to 55 mM per liter of cell water.

treated with saponin as it did in the frozen blood. Nevertheless, in the former the organic phosphates became filtrable, whereas in the latter they did not.

As far as potassium and sodium are concerned, the experiments give less clear cut information. The results are less consistent and less striking. There can be no doubt, however, that the behavior of the two elements is quite different; and this difference must signify that at least a fraction of one or both elements is under restraint. Again the experiments with whole blood prove that it is not this restraint which determines their disparate distribution between cells and serum. For this the cell membrane must still be held responsible. The chemical restraint only conditions or modifies their osmotic activity. Although at 7° some sodium and potassium is transferred across the cell membranes, the quantities are small; even at 37° distribution coefficients do not approximate those found in hemolyzed blood. The distribution coefficients of the two bases in the latter suggest that potassium plays a major rôle in combining with protein. The difference between the distribution coefficients of sodium in frozen chilled blood and in incubated or saponin-treated blood suggests that sodium may be associated with organic phosphates. To draw any such exact conclusions from these rough preliminary observations, however, would be hazardous in the extreme.

SUMMARY

1. When blood is hemolyzed by repeated freezing or by the addition of saponin, glycolysis and hydrolysis of phosphate esters proceed at a slower rate than they do in intact blood, but are not abolished.

2. When blood hemolyzed by these methods was ultrafiltered through cellophane, phosphates, sodium, and potassium did not distribute themselves between the water of ultrafiltrate and substrate as if they were restrained only by the Gibbs-Donnan equilibrium. (*a*) Under all conditions the concentration of inorganic phosphate was higher in the water of substrate. (*b*) Potassium distributed itself always according to the dictates of the Gibbs-Donnan equilibrium. (*c*) In blood hemolyzed by freezing, after 18 hours at 7°, the acid-soluble organic phosphorus remained unchanged and acted as if it was completely non-

filtrable. Under the same conditions the concentration of sodium was higher in the substrate. (*d*) In blood hemolyzed by saponin, after 18 hours at 7°, acid-soluble organic phosphorus, though intact, distributed itself equally between the water of substrate and ultrafiltrate. Sodium also appeared to be uniformly distributed. (*e*) In blood hemolyzed by freezing, after 18 hours at 37°, acid-soluble organic phosphorus was largely converted to inorganic phosphate, but the residual organic phosphate was distributed evenly throughout the water of substrate and filtrate. Sodium also appeared to be uniformly distributed.

3. The action of the same solutes in intact blood kept 18 hours at 7° and 37° was investigated. (*a*) At 7° organic acid-soluble phosphorus remained unchanged, imprisoned in the cells. A small amount of potassium escaped from the cells, while an equivalent amount of sodium passed in the reverse direction. (*b*) At 37° a large proportion of the organic acid-soluble phosphorus was broken down and much of the inorganic phosphorus derived from it passed into the serum, but no organic phosphate appeared to escape from the cells. A small amount of potassium left the cells and a far larger amount of sodium moved in the reverse direction.

4. The implications of these experiments with respect to the state of phosphates, potassium, and sodium in the red blood cells are discussed.

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THE EFFECT OF IODINE AND MERCURY ON AMINO NITROGEN VALUES WITH NITROUS ACID*

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In a recent paper, Dunn and Porush (1) report partial confirmation of our (2) findings on the effects of iodine on amino nitrogen values by Van Slyke's manometric method (3). They could not confirm our observation that glycine gives theoretical values when iodide is added, for they found the same values (103 per cent of theoretical) with as without iodide.

Since Dunn and Porush found theoretical values for amino nitrogen for cystine and cysteine by the use of iodide, and non-theoretical results with glycine, they postulated a new theory for the action of the iodide. This theory involves the formation of a complex compound of mercuric iodide with certain amino acids, especially cystine and cysteine, which in some way prevents the formation of extra nitrogen. According to their theory, this complex is not formed with glycine.

Although Dunn and Porush were unable to isolate their postulated complex mercury compounds of cystine or cysteine, they advanced other evidence for their existence. They claim that in the volumetric apparatus, where no mercury is present, the amino nitrogen values of cystine are the same (112 per cent of theoretical) in the presence as in the absence of iodide, while addition of K_2HgI_4 makes the values approximately theoretical. From values on the more positive oxidation potential of nitrite as compared with iodine, and also from the fact that iodine does not form sulfate from cystine (while nitrous acid readily does), they argue logically that our theory ((2) p. 164), which says that

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the iodine oxidizes these abnormal groups in deaminizing cystine and glycine so that these groups do not react with nitrous acid to form extra nitrogen, cannot be correct. They also claim that iodine has no effect on the amino nitrogen value of glutathione.

We do not agree with Dunn and Porush with respect to two simple experimental facts: first, concerning the effect of iodide on the manometric amino nitrogen value of glycine; and second, concerning the effect of iodide on the volumetric amino nitrogen value of cystine. As reported in our original paper (2), we continue to find that, while the amino nitrogen value of glycine is 103 to 104 per cent by Van Slyke's manometric method without iodide, in the presence of iodide the amino nitrogen value is 100 per cent. This has been observed independently by four different analysts on four lots of glycine from widely different sources. We also find that in the volumetric apparatus (absence of mercury) the addition of 1 cc. of 2 per cent KI to the reaction mixture has a similar effect, lowering the amino nitrogen value of glycine from 106 per cent of theoretical without iodide (Van Slyke's (4) procedure—5 minutes shaking at 24°) to 100 per cent with iodide. These and other data are summarized in Table I.

TABLE I

Amino Nitrogen Values for Cystine and Glycine

The figures are the per cent of the theoretical values without iodide. Corresponding values with iodide were 100 per cent throughout.

	Manometric		Volumetric			
			No mercury		With 0.3 cc. mercury	
	5 min.	10 min.	5 min.	10 min.	5 min.	10 min.
Glycine	103	107	106	107	103	
Cystine	130–150	145–155	107–110	113–116	117	125

Also, contrary to Dunn and Porush, we find that the amino nitrogen value of cystine in the volumetric apparatus (absence of mercury) is decreased from 108 per cent without iodide to 100 per cent with iodide. For these analyses the volumetric apparatus as modified by Koch (5) was used. The iodide used here, either 2 per cent KI in 97 per cent acetic acid, or 0.5 per cent KI in the

30 per cent $NaNO_2$, amounts to 0.26 per cent KI in the final reaction mixture and this is the same final concentration as that used in the manometric method. The volumetric amino nitrogen value of cystine without iodide is difficult to define precisely, because the result varies considerably with variations in the time and temperature of the reaction and increases considerably with traces of mercury salts. But the marked effect of iodide is invariable: the amount of N_2 formed is within 1 per cent of theoretical, it is not increased by doubling the time or increasing the temperature by 5°, and, as long as a suitable excess of iodide is present, it is not influenced by the presence of mercury salts.

Glycylglycine and glutathione were analyzed manometrically for amino nitrogen with and without KI, before and after hydrolysis, and the data are given in Table II. The glycylglycine, obtained from Amino Acid Manufactures at Los Angeles, showed 102.4 per cent of theoretical total nitrogen; the glutathione from the Eastman Kodak Company showed total N of 99.6 per cent and total carbon of 101.2 per cent of theoretical. The total nitrogen estimations were made according to Van Slyke (7) and the carbon according to Van Slyke, Page, and Kirk (8). The amino nitrogen values on glycylglycine, although not theoretical in the presence of KI, are markedly less than in the absence of KI. The values on glutathione are in substantial agreement with those previously reported (9) and are contrary to the statement of Dunn and Porush ((1) p. 268), "that amino nitrogen from glutathione is unaffected by the presence of iodide in the deaminizing reagent."

TABLE II

Per Cent of Theoretical Amino Nitrogen Values (Manometric) at 25°

	Without KI		With 2 per cent KI		With 20 per cent KI	
	3 min.	20 min.	3 min.	20 min.	10 min.	20 min.
Glycylglycine	136.7	144.2	120	127.2	127.4	126.9
Glutathione	204.8	229.7	193.2	199.0		195
Hydrolyzed* glycylglycine						101.7
" * glutathione	115.6	129.4	96.6	98.8		97.7

* Hydrolysis was carried out with 3 N HCl in a sealed ampule for 1½ hours at 15 pounds steam pressure and the HCl neutralized with NaOH (6).

As expected, the hydrolyzed peptides show very close to theoretical amino nitrogen values when KI is added.

The effect of metallic mercury in the volumetric method was studied by placing 0.3 cc. of metallic mercury in the volumetric chamber before the other reagents are added. The results (see Table I) are essentially the same as those by the manometric method. Without iodide the values for cystine are markedly increased, while those for glycine are decreased from 106 per cent without mercury to 103 per cent with mercury. The fact that glycine gives lower values, while cystine gives higher values by the manometric than by the volumetric (no mercury) method, is apparently an effect of the mercury in the former method. But the effect of iodide is invariably to lower the amino nitrogen value to theoretical with both amino acids, either with or without metallic mercury.

The effects of mercury salts on the amino nitrogen values of cystine and glycine were studied by using glacial acetic acid containing 5 per cent mercuric acetate. The results are qualitatively similar to those with metallic mercury—increase with cystine and decrease with glycine; quantitatively, however, the mercuric acetate decreases the glycine value to theoretical, while the metal leaves it at 103 per cent. In the presence of both mercuric salt and iodide, glycine gives theoretical values, as long as either one is present in sufficient excess. With *cystine*, however, the result depends upon which one is in excess. If the iodide is present in sufficient excess (final concentration of 0.6 per cent mercuric acetate and 1.1 per cent potassium iodide), the amino nitrogen value is theoretical. With an excess of mercuric salt (final concentration of 0.6 per cent mercuric acetate and 0.26 per cent potassium iodide) the N_2 values are as high as they are with mercuric acetate without any iodide.

Studies were made on the effect of time, temperature, and concentration of reagents on the amino nitrogen values of cystine and glycine, without and with iodide. Table I shows that as the time is increased from 5 to 10 minutes (at 20–25°) the nitrogen evolved from both cystine and glycine without iodide is definitely increased, while in the presence of iodide the values remain at 100 per cent in both times. Also as the temperature is increased from 20° to 30° with constant reaction time of 5 minutes, the N_2

evolved increases by 2 to 5 per cent without iodide, but remains constantly at 100 per cent in the presence of iodide. Similarly, as the concentration of reagents is doubled, the N_2 values increase by 4 to 8 per cent without iodide, while in the presence of iodide the values are increased by not more than 1 per cent. Thus in the manometric apparatus without KI when the reaction mixture consists of 5 cc. of 0.01 M glycine + 1 cc. of glacial acetic acid + 2 cc. of $NaNO_2$ (total volume 8 cc.), the N_2 evolved is 103 per cent of theory in 5 minutes at 20°; but when 1 cc. of 0.05 M glycine, 1 cc. of glacial acetic acid, and 2 cc. of $NaNO_2$ (total volume 4 cc.) are mixed, the N_2 evolved is 111 per cent. With cystine in one experiment the result was 148 per cent at 8 cc., and 155 per cent at 4 cc. total volume, the N_2 evolved without iodide being definitely greater at a higher concentration of reacting substances. But in the presence of iodide, cystine yielded the same 100 per cent value at both concentrations, while glycine, yielding 100 per cent at 8 cc. volume, gave 101 per cent at 4 cc. volume. The theoretical values obtained for these two amino acids in the presence of iodide is not due to a fortuitous balancing of errors, but the values hold under a wide variety of conditions of time, temperature, and concentration of reagents.

We have also observed that free iodine in the absence of iodide has the same effect as an equivalent concentration of iodide on the amino nitrogen values of both cystine and glycine in the volumetric as well as the manometric apparatus in the absence as well as in the presence of metallic mercury.

Van Slyke (4) found that carbon dioxide is formed when nitrous acid reacts with glycine and glycylglycine. We thought that a quantitative study of the CO_2 formed during the nitrous acid reaction with a variety of amino acids and peptides, in the presence and absence of iodide, of iodine, and also of metallic mercury, and of mercuric acetate, and a comparison of the carbon dioxide values with the corresponding amino nitrogen values might provide facts which would allow a better understanding of the mechanism of the reaction.

For the quantitative estimation of the carbon dioxide formed during the nitrous acid reaction, we used essentially the procedure of Van Slyke, Page, and Kirk for microdetermination of carbon (8). The nitrous acid reaction was carried out in the evacuated

combustion tube at room temperature and the carbon dioxide formed was absorbed in the chamber by 2 cc. of carbonate-free alkali, as described in the manometric microcombustion method.

Detail of Procedure for Determining CO_2 Formation during Nitrous Acid Reaction—Into the combustion tube are introduced 5 cc. of 0.01 N amino acid (or protein hydrolysate containing an equivalent amount of primary amino nitrogen) together with one of the following four other additions: 1 cc. of glacial acetic acid, 1 cc. of 2 per cent KI in 97 per cent acetic acid, 1 cc. of water + 1 cc. of glacial acetic acid, or 1 cc. of 20 per cent KI in water + 1 cc. of glacial acetic acid. Each of these mixtures was studied without and with the addition of 2 cc. of metallic mercury. Before the nitrite is added (see below), the solutions are freed from dissolved gases as follows:

The combustion tube containing amino acid and acetic acid, with or without iodide and mercury, is attached to the Van Slyke-Neill chamber through the connecting tube with the stop-cock, and grease is used at all joints. The tube is then evacuated by lowering the mercury in the reaction chamber. While in the evacuated condition, the combustion tube is shaken for ½ minute to remove physically dissolved CO_2. The air is then ejected through the cup of the reaction chamber. This procedure is repeated ten to fifteen times until all the air is removed, the combustion tube being shaken with each evacuation. 2 cc. of carbonate-free alkali are then introduced into the reaction chamber. The mercury in the chamber is lowered about half-way and the stop-cock of the chamber is left open to the combustion tube. 2 cc. of sodium nitrite are then introduced through the stop-cock of the connecting tube with a Van Slyke-Ostwald pipette. The combustion tube is then shaken for 3 minutes by hand. Then during the next 7 minutes the leveling bulb is raised and lowered, in order to insure complete absorption of the CO_2 by the alkali in the chamber, as described by Van Slyke, Page, and Kirk (8), and then the NO-N_2 gas in the chamber is ejected without loss of liquid through the stop-cock at the top of the reaction chamber. The leveling bulb is lowered again and the combustion tube is shaken for ½ minute; then the leveling bulb is raised and lowered for 4½ minutes more to get the last of the CO_2 over into the alkali. The connecting tube is detached from the reaction chamber and

the capillary of the side arm of the stop-cock is sealed with mercury. The NO and N_2 are then ejected through the cup without loss of liquid and then 1.5 cc. of normal lactic acid are measured into the chamber and the procedure of Van Slyke, Page, and Kirk is followed; the CO_2 is finally absorbed by 0.2 cc. of 5 N NaOH. The blank is determined in the same way as the analysis, except that water is used in place of the amino acid solution. The

TABLE III

CO_2 Formation from Reaction of Amino Acids with HNO_2 in 15 Minutes at 22–30°

5 cc. of 0.01 N amino acid + other addition indicated + 2 cc. of saturated $NaNO_2$.

Other addition	Mole CO_2 observed divided by equivalent of primary amino N				
	Glutamic acid	Glycine	Cystine	Glycylglycine	Glutathione
1 cc. glacial acetic acid	0.022	0.51	0.15	0.11	
1 " " " " + 2 cc. mercury	0.015	0.48	0.40	0.11	0.05
1 " " " " + 1 " water		0.45	0.15		
1 " " " " + 1 " " + 2 cc. mercury		0.40	0.40		
1 cc. 5% mercuric acetate in glacial acetic acid		0.42	0.42		
1 cc. 2% KI in 97% acetic acid	0.012	0.35	0.41	0.04	0.12
1 " 2% " " 97% " " + 2 cc. mercury	0.004	0.37	0.40	0.03	0.12
1 cc. 2% iodine (no iodide) in glacial acetic acid		0.40	0.46		
1 cc. glacial acetic acid + 1 cc. 20% KI in water	0.003	0.23	0.25	0.02	
1 cc. glacial acetic acid + 1 cc. 20% KI in water + 2 cc. mercury	0.002	0.25	0.23	0.01	0.14

moles of CO_2 formed are calculated from the pressure differences in the usual way, and the results are expressed in Table III in terms of the ratio of moles of CO_2 formed divided by equivalents of primary amino nitrogen.

Table III shows that the CO_2 formed from glycine and cystine (15 to 50 per cent of the amino N) is much greater than that from glutamic acid (2 per cent of the amino nitrogen or less). The

amount of CO_2 formed from glycine is decreased by the presence of either KI or Hg, while that of cystine is increased by these additions. The CO_2 formed from glycylglycine and from glutathione (per unit amount of primary amino nitrogen) is less than that obtained with the amino acids, cystine and glycine, but that for glycylglycine (like that of glycine) is decreased by iodide, while that of glutathione (like that of cystine) is increased by iodide. The CO_2 formation from protein hydrolysates (after removal of ammonia) is 0.22 to 0.33 of the primary amino nitrogen and is decreased to 0.19 to 0.24 by 2 per cent KI, and to 0.11 to 0.20 by 20 per cent KI; gelatin and hair hydrolysates show larger values and greater decreases with KI than hydrolysates of egg albumin or casein. The protein hydrolysates were prepared by autoclaving with 3 N HCl for 3 hours (6), and the ammonia was removed by vacuum distillation with $Ca(OH)_2$. The amount of CO_2 formed from cystine or glycine during the nitrous acid reaction varies considerably with variation in the concentration of reagents. In one case in which 1 cc. (instead of the usual 5 cc.) of glycine solution was mixed with glacial acetic acid and nitrite, the CO_2 formed was 0.7 mole per equivalent of amino N instead of 0.51, as given in Table III. In all cases reported in Table III the concentration of reagents was carefully controlled with a total volume of 8 or 9 cc., as indicated above.

DISCUSSION

The theory of Dunn and Porush that the effect of iodide on the manometric amino nitrogen value of *cystine* is dependent upon a complex mercury compound with iodide and amino acids is excluded, since in the volumetric apparatus, in the complete absence of mercury or its salts, iodide lowers the amino nitrogen value. Further, the statement of Dunn and Porush that iodide has no effect on the amino nitrogen value of *glycine* is definitely contradicted by our repeated finding, in both volumetric and manometric apparatus, that adding iodide lowers the amino nitrogen value to theoretical. Further, we find that mercuric acetate (without and with iodide) also lowers both the volumetric and manometric glycine value to theoretical. On cystine, however, mercuric acetate has the opposite effect, raising the value to 125 to 140 per cent; and even in the presence of iodide, with

mercuric acetate in excess (0.25 per cent KI, 0.6 per cent $Hg(OAc)_2$), the amino nitrogen value of cystine remains abnormally high. These facts likewise contradict the theory of Dunn and Porush which would prescribe that the glycine value should remain unaffected, while that of cystine should be lowered.

So far we see no reason to modify our theory ((2) p. 164) which can be stated more specifically as follows: Iodine, formed from oxidation of iodide by nitrous acid, oxidizes the abnormal groups in deaminizing cystine and glycine before the nitrous acid can do so, and so, by preventing the reduction of nitrous acid by the abnormal reducing groups, the iodide prevents the formation of extra nitrogen. The facts about CO_2 formation, and the effects of mercuric salts, complicate the picture somewhat and demand special assumptions, but none of these facts contradicts the original idea that the iodide acts through the oxidizing action of iodine.

We believe that on *glycine* mercuric acetate acts in the same way as iodine does, to oxidize the abnormal group before the nitrous acid does. With *cystine*, however, mercuric acetate not only is unable to prevent the reduction of the nitrous acid by the abnormal group, but it promotes this reduction with formation of increased amounts of extra nitrogen. This does not mean that iodine does not oxidize the deaminizing cystine. Added iodide and mercuric acetate both cause about the same increase in CO_2 formed from cystine plus HNO_2, indicating that both act to about the same extent in promoting the oxidation of the carbon chain. But the iodine acts to prevent the extra reduction of the nitrous acid, while the mercuric acetate must form some intermediate which promotes this extra reduction; whereas both act to cause increased CO_2 formation.

An unexpected fact is that both iodine and mercuric acetate decrease slightly, rather than increase, the CO_2 formed from *glycine* with nitrous acid, while preventing the formation of extra nitrogen. Although these reagents obviously act to decrease slightly the extent of oxidation of the carbon, the amount of CO_2 formed from glycine even in the presence of iodine or mercury is much greater than that formed from a "normal" amino acid such as glutamic acid. We do not feel that this argues against our theory of the oxidative action of iodine. Rather we feel that it is the *iodide* (possibly by its reducing action) which decreases the formation

of the CO_2 while the oxidative action of the iodine prevents the formation of extra nitrogen. At first thought it might seem that this idea is excluded by the observation that free iodine, in the complete absence of added iodide, has essentially the same effect as iodide on the CO_2 as well as on the N_2 value. However, when iodine oxidizes, iodide is formed, and the effects of added iodine on CO_2 formation might well be the result of iodide secondarily formed by reduction of iodine. This view is supported by several facts. Although added iodine decreases the CO_2 formed as compared with no iodine, the CO_2 value with iodine is somewhat greater than that with iodide. Also 20 per cent KI decreases the CO_2 from glycine even more than 2 per cent KI, while leaving the N_2 theoretical; and with cystine, 20 per cent KI, while increasing the CO_2 above the value without iodide, leaves it at a lower value than 2 per cent KI. We believe that the *iodine* controls the N_2 formation by oxidizing the abnormal groups in deaminizing glycine, thus preventing the extra reduction of nitrous acid, while iodide, either originally present or formed from iodine by the reduction described above, decreases in some way the formation of CO_2 from deaminizing glycine.

Dunn and Porush argue logically that, since nitrous acid has a higher oxidation potential than iodine, the iodine could not preferentially oxidize any reducing group in the presence of nitrous acid. The whole subject of oxidation catalysis is replete with examples of weak oxidizing agents, of intermediate or low potential, promoting oxidations which substances of higher potential cannot effect in a limited time. Thermodynamic constants allow us to predict the direction *but not the rates* of chemical reactions. We believe that iodine may well oxidize a reducing group in deaminizing cystine or glycine, which the nitrous acid with higher potential cannot oxidize in the same time just as, for example, iodine at pH 6 rapidly oxidizes hydroquinone which oxygen with higher potential cannot oxidize except very slowly.

Dunn and Porush also point out that, while nitrous acid (by oxidation) forms sulfate from cystine, iodine, in the absence of nitrite, is unable to do so. This fact we have confirmed, but we do not feel that it bears on the question. Van Slyke (4) showed that glycolic acid yields no N_2 with nitrous acid and Lough and Lewis ((10) p. 608) showed this similarly for β,β'-dithiodiglyceric acid. We feel that it is probably not the original amino acid,

nor the amino acid after complete deamination, but rather the amino acid during deamination, possibly a diazo intermediate, which forms the extra nitrogen. Cystine and glycine which are being deaminized by nitrous acid probably have quite different reactivities than have the amino acids without nitrous acid; diazo compounds usually have greater reactivity than their parent amines or the resulting alcohols. We feel that it is impossible to predict from the lack of reaction of iodine with pure cystine that there will be a similar lack of reaction between iodine and deaminizing cystine.

We attach no particular significance or permanence to our theory. Like most working hypotheses, it will probably be amplified or entirely superseded by better ideas. We defend it here only because it has been unfairly attacked and because we feel that it is still the best interpretation of the available facts.

Change in Manner of Adding Iodide—In our early work on the effect of iodide on amino nitrogen values (2), the iodide was dissolved in and added with the acetic acid. This solution of 2 per cent KI in 97 per cent acetic acid is rapidly oxidized when exposed to air and in a few hours it becomes intensely colored with free iodine. Since a solution prepared by dissolving 2 per cent of iodine (no iodide) in acetic acid has the same effect on amino nitrogen values, we concluded that the atmospheric oxidation of the iodide in the KI solution could do no harm, and that the reagent would be stable indefinitely.

More recently, however, have we found that such solutions, after aging for several weeks, at times have decreased efficiency in lowering the amino nitrogen values of cystine to theoretical. The reason apparently is that the iodine reacts with mercury during the preliminary shaking for removal of dissolved air, mercuric iodide is formed, and the net amount of dissolved iodine during the nitrous acid reaction is decreased. This difficulty can be increased by prolonging the time of the preliminary shaking to 5 instead of the usual 2 minutes, and especially by allowing a few globules of mercury to remain with the aqueous phase in the wide portion of the chamber during this preliminary shaking. Under this latter condition, all the brown iodine color is completely discharged, and, in case the acetic acid contained much free iodine and little or no iodide, a heavy yellow precipitate of mercuric iodide is formed. When subsequently the nitrite is added, very

little or no free iodine is formed, and the amino nitrogen values of cystine or glycine are higher than theoretical, as they are without any iodide or iodine. This difficulty is never observed with fresh solutions (less than 2 days old) of KI in acetic acid, in which a large fraction of the iodide remains unoxidized and it also can be avoided with old, extensively oxidized solutions of iodide in acetic acid, or even with solutions made by dissolving free iodine without iodide directly in acetic acid, if the time of the preliminary shaking is not allowed to exceed 2 minutes, and if the contact of mercury with the aqueous phase during this preliminary shaking is kept at a minimum.

The difficulty can, however, be more conveniently overcome by dissolving the iodide in, and adding it with, the nitrite. For the manometric method, since 2 cc. of nitrite are used and 20 mg. of KI are wanted, one dissolves 1 gm. of KI in 100 cc. of saturated $NaNO_2$ (800 gm. of $NaNO_2$ + 1 liter of water as described by Van Slyke (3)). In the volumetric method, one uses 0.5 gm. of KI in 100 cc. of the 30 per cent $NaNO_2$ (where 3 cc. of glacial acetic acid and 12 cc. of 30 per cent $NaNO_2$ are used with a 1 or 2 cc. sample of amine). The iodide readily dissolves in the nitrite; there is no atmospheric oxidation of iodide and the solution keeps indefinitely.

A further advantage of this iodide-nitrite solution in the manometric method arises from the fact that some proteins form sticky precipitates when treated with iodine in acetic acid, but not with acetic acid alone; and when these precipitates adhere to the constricted portions of the chamber above the 2 or 0.5 cc. marks, they are difficult to remove and cause inaccuracies in the analysis. When the iodide is added with the nitrite, the mixture of amine and acetic acid, after removal of dissolved gases, can be drawn well below the 2 cc. mark before the iodide-nitrite is added, and the protein precipitates adhering to the wide portion of the chamber not only do not interfere with the gas volume above the 2 cc. mark, but they are readily removed by subsequent shaking with dilute alkali.

SUMMARY

1. By the addition of iodide, the nitrous acid amino nitrogen values of cystine and glycine become theoretical by the volumetric

as well as the manometric method, in the absence as well as in the presence of metallic mercury.

2. Mercuric acetate lowers the amino nitrogen value of glycine to the theoretical, while metallic mercury lowers it from 106 to 103 per cent. With cystine, mercuric acetate and metallic mercury cause marked increases from 108 to 140 per cent or more.

3. Cystine and glycine form considerable CO_2 during the nitrous acid reaction, while the CO_2 from glutamic acid is nearly zero. This CO_2 from glycine is lowered slightly by added iodide or mercuric acetate, while the CO_2 from cystine is considerably increased by such additions.

4. For interpretation we still prefer the theory that added iodine oxidizes some abnormal reducing groups in deaminizing cystine and glycine, and thus prevents the reduction of nitrous acid by these groups which otherwise cause formation of extra nitrogen.

5. For both volumetric and manometric analyses with iodide, it is recommended that the KI be dissolved in and added with the nitrite instead of the glacial acetic acid.

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THE DETERMINATION OF TOTAL BASE IN BIOLOGICAL MATERIAL BY ELECTRODIALYSIS*

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The microdetermination of total base by electrodialysis has been subjected to further investigations since the communication of Keys (1) and of Joseph and Stadie (2). An improved total base unit[1] has been devised which is easy to manipulate and which allows increased efficiency and greater accuracy in the determination. In this communication the use of the new unit will be discussed and a critical comparison will be made between determination of total base by electrodialysis and by other procedures in common use.

Reagents and Apparatus

Instrument—Several parts of the unit described by Keys have been eliminated. An instrument with nine improved units[2] is shown in Fig. 1. This includes two milliameters, a pilot lamp, a rheostat, a shunt, and a switch. It is operated at 110 or 220 volts D.C.; the possibility of using lower voltages has not been tested fully. It draws 65 milliamperes at full load.

Anode—The anode is a platinum electrode fused in a Pyrex cup and connected by a metal socket directly to the source of power. The anode acid-conductor of Adair and Keys (3) has been eliminated. An unsuccessful attempt was made to substitute platinum foil for the mercury anode.

* This investigation was aided by the Corn Industries Research Foundation.

[1] Demonstrated at the meeting of the Federation of American Societies for Experimental Biology, Toronto, April, 1939.

[2] Manufactured by Macalaster-Bicknell Company, Cambridge, Massachusetts.

Anode cups should not be cleansed with cleaning solution. Precipitation of chromium on platinum may introduce serious errors. A detailed sketch of one unit is shown in Fig. 2. It is recommended that the cups be brushed with water, filled with concentrated nitric acid, and allowed to stand for several hours. Several rinses with distilled water remove all adherent base.

Membranes - Cellophane (No. 300 plain transparent cellophane, du Pont) is the most satisfactory material for membranes we have discovered. A 7 cm. square, after soaking for 10 minutes or more in distilled water, is placed over the finely polished end

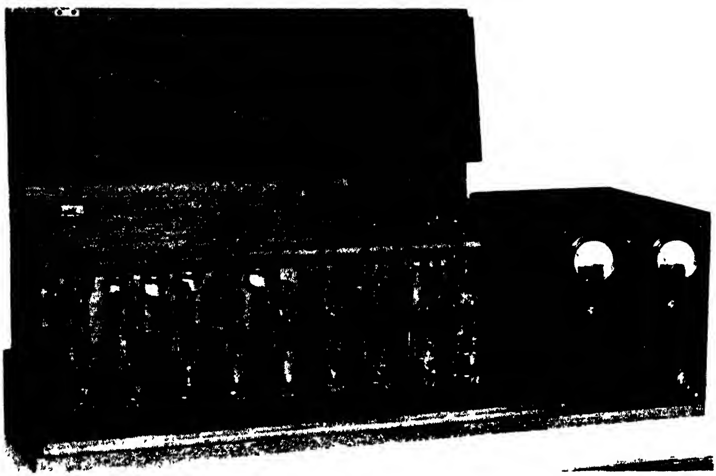

FIG. 1 Electrodialysis instrument. For details see the text

of the membrane tube. It is pulled taut with a slight downward pressure until all of the wrinkles have been removed. If the membrane is stretched across the grain before application, a better result is obtained. After application the maximum pull should be against the grain. The membrane is allowed to dry and trimmed with a razor blade or scalpel, leaving a 5 mm. overlap on the glass. The sides are sealed with collodion[3] by mechanical rotation of the membrane tube in a horizontal position, at 60 to 120 R.P.M. A thin continuous stream of collodion is poured onto the tube, which is begun 2 cm. away from the trimmed edge and

[3] Mallinckrodt U.S.P. collodion proved satisfactory.

worked out to the end of the tube. The membranes are dried, rinsed thoroughly, and stored in distilled water in the cold until ready for use. Membranes are used only once, since they become fragile and dirty.

Base-Free Water—Inexpensive base-free water may be obtained by redistillation from alkaline permanganate in an all-glass still. The first quarter of the distillate should be discarded. Pyrex bottles should be used for storage.

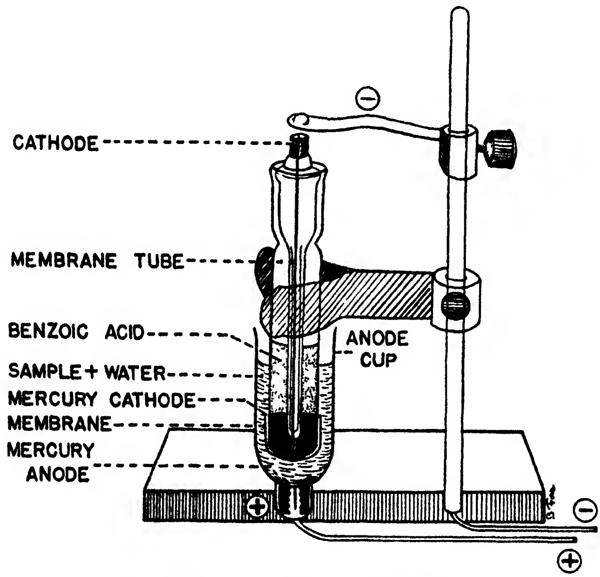

FIG. 2. Sketch of one electrodialysis unit

Pure Mercury—Used mercury is cleansed by bubbling air through the mercury container to which has been added 5 per cent nitric acid, and finally rinsed thoroughly with distilled water.

Standard Benzoic Acid—Benzoic acid has been substituted for the stronger mineral acids. It is preferable for electrodialysis, as it may be employed with either an organic or an inorganic base. A stable product (Mallinckrodt) which assays about 99.95 per cent is available. A stock solution of N benzoic acid is prepared in absolute alcohol and stored in the cold. A 0.02 N solution is made from stock by diluting with glass-distilled water. We have

used one stock solution for more than 3 years and have detected no change in titer in the interim.

Standard Sodium Hydroxide—A solution of 0.2 N sodium hydroxide is prepared from carbonate-free sodium hydroxide.

Pipettes and Burettes—The Krogh-Keys (4) automatic syringe pipettes[2] of 0.2 ml. and 2.0 ml. capacity and the Rehberg microburette (5, 6) are indispensable.

Blank Analyses—The blank has been a source of unexplained trouble. If an individual blank is determined for reagents used, an error as great as ± 3.0 milliequivalents per liter of base may be introduced. A standard salt solution, on the other hand, gives reproducible results. We substituted for the blank analysis, therefore, 0.2 ml. of a solution of sodium chloride which was known to contain 140.0 milliequivalents per liter. The blank correction was computed from the recovery of base in this sample.

Procedure

Serum—Approximately 0.3 ml. of pure mercury and 10 ml. of glass-distilled water are placed in the anode cup. 0.2 ml. of serum is delivered into the cup with the automatic pipette. The pipette is rinsed ten times with glass-distilled water. In a newly rinsed membrane tube are placed approximately 1.5 ml. of pure mercury and exactly 2.0 ml. of standard 0.02 N benzoic acid. The cathode membrane is immersed in the unknown to a depth of 5 mm. Electrodialysis is started and allowed to proceed with maximum resistance for an hour. The resistance is cut out and electrodialysis is continued for about 12 hours, preferably through the night. The current flow should be negligible at the end of this period. The electrode is rinsed with a few ml. of glass-distilled water and a drop of uniform size of 0.04 per cent phenolphthalein added. The solution is titrated to the first trace of pink with 0.2 N sodium hydroxide from the microburette.

Complete dialysis of base from serum is achieved by 1 unit in 15 minutes. The time for 9 units is approximately 3 hours. For urine and tissues, a longer time is needed to allow for diffusion of calcium and magnesium. Electrodialysis overnight is adequate for all bases and avoids interfering ammonia fumes present in many laboratories during the day.

Blood Cells—Erythrocytes should be analyzed soon after col-

lection. A 1 mm. bore needle on an automatic syringe pipette is recommended for the transfer. 0.2 ml. of cells is used and the syringe is rinsed as for serum. 1.5 ml. of standard benzoate are sufficient. In order to free any occluded base, it is advisable to agitate frequently the protein which clumps on the membrane.

Urine—Urine is collected immediately after voiding, preserved with toluene, and stored at 4°. 24 hour urine specimens are diluted to 2 or 3 liters. With these precautions there is no precipitation of phosphates and urates and no appreciable amount of ammonia formation. In the determination of total base it is necessary to guard against an excessive amount of material. The sample should contain less than 0.04 milliequivalent of base.

Tissue—Complete recovery of base from minced tissue, ashed tissue, or sliced tissue is achieved if overnight dialysis is employed. 0.25 gm. of tissue is the maximum amount that may be taken. It is convenient to weigh 0.2 gm. of tissue directly into a clean, dry anode cup. 12 ml. of water and 0.3 ml. of mercury are added. Analysis proceeds as with serum.

Miscellaneous Samples—Plasma, whole blood, ultrafiltrates, and spinal and synovial fluid are treated in the same way as serum. They may be preserved at 4° for at least 3 weeks without any appreciable decomposition. The difficulty of obtaining a small homogeneous sample of dried diets or feces has prevented the application of the method to these substances. The high magnesium content of feces further complicates the analysis of this material. Not more than 0.005 milliequivalent of magnesium can be recovered from an unknown or standard solution if other bases are present.

Ashed Material—It is unnecessary to ash most samples of biological material. In the treatment of certain substances in which this is unavoidable, a dry ashing technique is employed. Material containing approximately 0.05 milliequivalent of base is dried in a 5 ml. platinum crucible or a quartz tube. The crucible is placed in a cold electric muffle furnace and ashed overnight at a temperature between 450–500°. The temperature is raised slowly. 1 drop of 0.2 N hydrochloric acid is added to the container when cool. An excess of acid is to be avoided, as it may interfere with the passage of anions through the membrane. After the crucible is agitated to accelerate solution of the oxides

of calcium and magnesium, the contents are rinsed into the anode cup with six 2 ml. portions of glass-distilled water. 0.3 ml. of mercury is added and electrodialysis started.

Results

Individual bases as well as total base were determined on samples of serum from thirty-seven males. The subjects were either healthy persons or ambulatory patients suffering from diseases not associated with a disturbance of the acid-base balance of the blood. All bloods were collected the same day from 4 to 8 hours after the morning meal. All determinations are given in Table I; none is omitted. Sodium was determined according to the method described by Butler and Tuthill (7). The samples were dry-ashed in platinum crucibles. Potassium was determined according to the method described by Consolazio and Talbott (6), calcium according to the method described by Clark and Collip (8), and magnesium by a modification of the method described by Briggs (9). Total base was determined by electrodialysis with 0.2 ml. samples. The data given are the original single analyses. No analyses were repeated. The agreement between the sum of the ions determined individually and determined collectively is satisfactory. The average of the values for total base determined by electrodialysis was 153.1 milliequivalents per liter. The average of the sum of the bases determined individually was 152.5 milliequivalents per liter. The extra base obtained by electrodialysis may be attributed to small amounts of organic or inorganic base not determined usually.

Table II contains data of serum base of fifteen healthy males. The bases were determined individually as Na, K, and Ca; collectively as total base by a gravimetric method (10) on 20 ml. samples and total base by electrodialysis on 0.2 ml. The average total base as determined by electrodialysis was 0.7 milliequivalent per liter less than that for total base by the gravimetric method with 100 times the quantity of serum. The results are equally satisfactory if averages are taken of the first seven samples with individual bases analyzed. Magnesium was not determined in these samples but if 2.7 milliequivalents (the average for Table I) are added to the average for the individual bases the result is 154.0 milliequivalents per liter. The average for total base determined gravi-

TABLE I

Concentration of Serum Bases Determined Individually and Collectively

Concentrations are given in milliequivalents per liter.

Subject No.	Na	K	Ca	Mg	Σ	Total base by electro-dialysis	Total base minus Σ
1	138.5	4.77	5.20	2.70	151.2	151.4	+0.2
2	141.9	4.63	5.10	3.15	154.7	154.5	−0.2
3	137.7	4.62	4.72	3.05	150.1	147.7	−2.4
4	143.0	4.40	4.85	2.87	155.1	153.7	−1.4
5	139.6	4.11	4.96	2.48	151.1	151.5	+0.4
6	141.8	3.88	4.90	2.63	153.2	153.1	−0.1
7	143.0	4.00	5.16	2.66	154.8	152.5	−2.3
8	141.1	4.83	4.98	2.52	153.4	152.9	−0.5
9	139.0	4.45	4.94	2.21	150.6	150.8	+0.2
10	140.3	4.38	5.09	2.54	152.3	152.9	+0.6
11	140.5	5.13	4.98	2.97	153.6	154.3	+0.7
12	142.2	4.72	4.67	2.63	154.2	154.7	+0.5
13	141.6	4.77	5.13	2.48	154.0	153.9	−0.1
14	141.6	4.15	4.86	2.44	153.0	152.9	−0.1
15	140.4	4.19	4.86	2.41	151.9	152.0	+0.1
16	140.5	4.30	4.91	2.41	152.1	152.4	+0.3
17	138.8	4.51	5.27	2.54	151.1	152.5	+1.4
18	141.0	5.16	4.92	2.30	153.4	155.3	+1.9
19	139.4	4.00	5.17	2.37	150.9	152.0	+1.1
20	138.4	5.17	5.06	2.44	151.1	152.4	+1.3
21	135.0	5.59	4.53	2.48	147.6	149.4	+1.8
22	141.0	4.46	5.15	2.39	153.0	154.4	+1.4
23	141.1	4.32	5.21	2.78	153.4	153.6	+0.2
24	141.5	5.08	4.73	2.63	153.9	154.8	+0.9
25	140.5	4.66	4.98	2.96	153.1	152.3	−0.8
26	141.2	4.56	5.05	2.54	153.4	153.7	+0.3
27	140.0	4.70	5.05	2.48	152.2	152.4	+0.2
28	139.0	4.24	5.01	2.63	150.9	152.6	+1.7
29	142.6	5.47	5.22	2.72	156.0	156.8	+0.8
30	139.7	4.27	5.01	2.63	151.6	152.9	+1.3
31	139.6	4.88	5.20	2.70	152.4	152.9	+0.5
32	141.0	4.29	4.87	3.35	153.5	155.3	+1.8
33	139.0	4.59	4.98	2.95	151.5	154.0	+2.5
34	139.6	4.45	4.76	2.59	151.4	153.8	+2.4
35	139.5	5.00	4.96	3.05	152.5	154.0	+1.5
36	139.7	4.79	5.35	2.97	152.8	152.8	0.0
37	138.5	4.40	5.21	2.97	151.1	153.2	+2.1
Average	140.2	4.60	5.00	2.67	152.5	153.1	+0.6

metrically in the same samples was 154.9 milliequivalents and for total base by electrodialysis 154.2 milliequivalents per liter.

In Table III data are presented of serum bases of thirteen hospital patients. The bases were determined individually as Na, K, and Ca; collectively as total base according to the method described by Van Slyke, Hiller, and Berthelsen (11), and total base by electrodialysis. If it is assumed that the average concentration of Mg is 2.7 milliequivalents, the sum of the bases determined

TABLE II

Concentration of Serum Bases Determined Individually As Na, K, and Ca, and Collectively by Gravimetric Method and by Electrodialysis

Concentrations are given in milliequivalents per liter.

Subject No.	Na	K	Ca	Σ	Total base gravimetrically	Total base by electrodialysis
38	140.8	3.4	5.2	149.4	152.3	152.4
39	140.1	3.3	5.3	148.7	153.7	151.6
40	143.9	4.0	5.1	153.0	156.1	156.1
41	140.5	3.7	5.4	149.6	154.3	153.1
42	142.5	3.9	4.9	151.3	152.6	152.6
43	143.6	4.0	5.1	152.7	154.6	153.9
44	146.3	4.1	5.0	155.4	160.8	159.1
45					155.4	154.9
46					153.5	152.4
47					154.5	154.4
48					157.2	155.7
49					152.0	152.0
50					152.2	153.0
51					151.9	150.9
52					154.0	152.5
Average					154.3	153.6

individually is 150.7 milliequivalents per liter. This agrees precisely with the average total base value by the method of Van Slyke *et al.* The average total base by electrodialysis was 150.4 milliequivalents per liter.

In Table IV, total base in thirteen samples of serum as determined according to the method described by Hald (12) is compared with total base determined by electrodialysis. The subjects were healthy males or females. Agreement of the data in Table IV is

unsatisfactory. The average difference is +5.6 milliequivalents per liter. The average total base which we obtained on serum from normal persons by Hald's method (147.6 milliequivalents) agrees with the average of ten determinations (146.5 milliequivalents) on normal persons in the author's article. It appears, therefore, that we were able to apply the technique as described. The disagreement between Hald's method and other micromethods has not been explained satisfactorily. We are inclined to believe

TABLE III

Concentration of Serum Bases Determined Individually As Na, K, and Ca, and Collectively according to Method Described by Van Slyke et Al. and by Electrodialysis

Concentrations are given in milliequivalents per liter.

Subject No.	Na	K	Ca	Σ	Total base by method of Van Slyke *et al.*	Total base by electrodialysis
53	142.5	4.7	6.4	153.6	154.0	156.4
54	142.8	3.5	4.9	151.2	153.7	154.0
55	143.5	3.1	5.7	152.3	155.7	154.9
56	137.5	14.1	5.1	156.7	159.0	158.8
57	141.7	4.1	4.9	150.7	150.0	153.0
58	142.5	3.2	4.6	150.2	153.5	153.2
59	119.0	5.0	4.7	128.7	128.8	130.2
60	136.2	4.7	4.8	145.7	151.3	150.2
61	137.9	4.1	4.5	146.5	150.0	148.4
62	136.2	4.4	4.4	145.0	150.5	147.9
63	141.2	4.8	4.8	150.8	153.5	151.2
64	140.5	4.1	4.6	149.2	153.2	151.9
65	134.9	4.5	4.5	143.9	146.5	145.3
Average	138.2	4.9	4.9	148.0	150.7	150.4

that Hald's method gives, consistently, results that are 4 to 6 milliequivalents too low.

Comparison was next made between concentration of total base of blood cells determined by the gravimetric method (10) and by electrodialysis (Table V). Samples of blood cells from eleven healthy males were used. 15 ml. of cells were employed for the gravimetric procedure and 0.2 ml. of cells for electrodialysis. Phosphates were removed as basic ferric phosphate in the gravi-

TABLE IV

Concentration of Serum Bases Determined As Total Base according to Method Described by Hald and by Electrodialysis

Concentrations are given in milliequivalents per liter.

Subject No.	Total base by Hald method	Total base by electrodialysis	Δ
66	144.8	153.2	+8.4
67	148.8	153.1	+4.3
68	152.2	156.7	+4.5
69	145.3	152.9	+7.6
70	145.5	152.2	+6.7
71	147.0	153.0	+6.0
72	148.2	151.6	+3.4
73	146.2	150.9	+4.7
74	149.6	152.5	+2.9
75	147.5	151.7	+4.2
76	151.9	156.5	+4.6
77	144.0	154.5	+10.5
78	148.0	152.8	+4.8
Average........	147.6	153.2	+5.6

TABLE V

Concentration of Cell Base Determined As Total Base by Gravimetric Method and by Electrodialysis

Concentrations are given in milliequivalents per liter.

Subject No.	Total base gravimetrically	Total base by electrodialysis	Δ
79	110.5	111.3	+0.8
80	107.5	111.2	+3.7
81	110.1	112.3	+2.2
82	112.6	111.7	−0.9
83	111.4	111.5	+0.1
84	113.5	113.5	0.0
85	112.7	112.5	−0.2
86	119.8	118.9	−0.9
87	116.3	114.8	−1.5
88	114.7	113.7	−1.0
89	111.6	110.5	−1.1
Average........	112.8	112.9	+0.1

metric procedure. The sample was then reashed with sulfuric acid and the sulfate weighed as the barium salt. The average discrepancy between the two methods was 1.0 milliequivalent per liter.

TABLE VI

Concentration of Bases in Urine Determined Individually As Na, K, Ca, Mg, and NH_4 and Collectively by Electrodialysis

Concentrations are given in milliequivalents per liter.

Urine sample No.	Na	K	Ca	Mg	NH_4	Σ	Total base by electro-dialysis	Total base minus Σ
1	69.4	37.8	0.3	7.2	27.8	142.5	144.8	+2.3
2	67.0	36.2	0.3	7.1	24.8	135.4	135.2	−0.2
3	72.4	32.7	0.1	7.2	26.0	138.4	138.0	−0.4
4	95.4	35.1	0.4	8.6	25.3	164.8	166.2	+1.4
5	85.2	36.7	0.3	8.0	24.6	154.8	155.8	+1.0
6	84.4	34.0	0.1	8.1	25.0	151.6	151.8	+0.2
7	79.8	32.4	0.3	8.0	23.4	143.9	142.6	−1.3
8	76.0	31.2	0.4	7.3	24.6	139.5	141.6	+2.1
9	94.8	36.2	0.6	8.5	26.3	166.4	167.3	+0.9
10	87.8	35.7	0.1	7.9	24.6	156.1	157.6	+1.5
11	75.0	33.7	0.3	7.0	27.4	143.4	143.6	+0.2
12	91.9	36.1	0.2	7.0	28.1	163.4	164.8	+1.4
13	69.8	35.4	0.3	6.0	26.6	138.1	136.8	−1.3
14	111.5	65.1	7.4	8.6	40.4	233.0	230.6	−2.4
15	112.8	64.2	7.7	8.6	42.9	236.2	235.8	−0.4
16	143.5	62.7	7.6	9.0	37.2	260.0	259.8	−0.2
17	136.2	65.3	7.7	9.3	44.7	263.2	263.0	−0.2
18	128.7	119.8	5.3	8.4	50.7	312.9	303.0	−9.9
19	94.2	135.6	4.9	9.6	49.7	294.0	284.6	−9.4
20	88.5	136.2	5.9	10.9	47.0	288.5	286.0	−2.5
21	187.0	317.8	10.3	9.0	34.0	558.1	562.0	+3.9
22	87.6	349.0	10.8	8.7	40.6	496.7	489.8	−6.9
23	62.4	356.8	11.4	8.4	38.2	477.2	479.6	+2.4

In Table VI data are given for base determinations in twenty-three samples of urine. The ions were determined individually as Na, K, Ca, Mg, and NH_4 and collectively as total base by electrodialysis. Agreement between the sum of the bases and total base by electrodialysis was 99 per cent or better in all except three samples. The greatest absolute discrepancies were noted in samples of urine with high concentrations of base. It is con-

cluded that the improved unit allows complete diffusion of bases occurring in urine and that recovery of base is complete when optimum quantities are taken for analysis.

During the past 3 years total base has been determined by electrodialysis in more than 1500 samples of serum and in more than 1000 samples of urine. Both normal and sick persons acted as experimental subjects. In a high percentage of instances the results by electrodialysis appeared to be satisfactory. The basis

TABLE VII

Concentration of Tissue Bases Determined Individually As Na, K, Ca, and Mg and Collectively by Electrodialysis

Concentrations are given in milliequivalents per kilo of tissue water.

Sample No.	Tissue	Na	K	Ca	Mg	Σ	Total base by electrodialysis	Total base minus Σ
1	Skin	91.3	14.7	5.1	16.3	127.4	136.6	+9.2
2	"	113.8	16.0	9.9	16.5	156.2	154.2	−2.0
3	"	90.4	19.2	9.4	19.0	138.0	144.2	+6.2
4	"	122.2	35.1	16.9	10.7	184.9	173.5	−11.4
5	"	140.7	25.9	10.7	11.5	188.8	192.0	+3.2
6	"	116.9	32.6	8.3	8.2	166.0	170.6	+4.6
7	"	118.8	13.3	6.0	14.0	152.1	152.0	−0.1
8	"	118.0	29.4	7.0	20.6	175.0	173.2	−1.8
9	"	102.7	44.2	8.7	9.0	164.6	168.6	−4.0
10	"	115.3	57.1	20.4	18.2	211.0	222.8	+11.8
11	"	188.5	55.2	14.9	13.4	272.0	271.0	−1.0
12	Liver	84.2	77.0	6.3	17.3	184.8	185.0	+0.2
13	"	93.1	97.4	4.7	20.5	215.7	223.0	+7.3
14	Muscle	55.6	109.7	6.1	18.1	189.5	189.0	−0.5
15	"	52.6	118.6	4.6	23.2	199.0	192.5	−6.5

of the anticipated results was clinical diagnosis, degree of disturbance of acid-base balance, or determination of base by other methods. Approximately ten samples of serum and urine from three patients, however, gave results which suggested the presence of an unidentified organic or volatile base other than ammonia. This conclusion was reached because the results of repeated determinations of total base by electrodialysis were considerably in excess of those that were determined by analysis of individual ions usually present. Several of the samples were ashed and total

base repeated by electrodialysis. The subsequent analyses agreed with the sum of the individually determined ions. The discrepancy in unashed specimens was observed in specimens from hospitalized patients only. It never was observed in the serum or urine from healthy persons.

Application of the improved unit to tissue analysis proved satisfactory, as is shown by the data for samples of normal and pathological tissues given in Table VII. All of the samples of skin were removed from living persons with the aid of local anesthesia. The samples of muscle and liver were obtained at autopsy. The base concentrations should not be interpreted as normal values, even though the tissues appeared normal, for all of the

TABLE VIII

Recovery of Base by Electrodialysis from Treated and Untreated Muscle

Concentrations are given in milliequivalents per kilo of tissue.

Sample No.	Untreated before analysis	Ashed before analysis	Minced before analysis
16	143.5	139.5	
17	115.3	114.5	
18	134.3	135.3	134.0
19	131.3	130.3	131.3
20	138.8	137.2	
21	141.6	139.5	
22	114.7	114.5	

patients were suffering from a metabolic disturbance. The several bases were determined individually and their summation compared with total base as determined by electrodialysis. Since it is difficult to sample tissue, the discrepancies in most instances can be charged to inadequacy of sampling.

It seems unnecessary to mince or to ash tissue before analysis. In Table VIII are presented data of analyses of muscle with and without special preparation. The experimental material was obtained with the aid of local anesthesia from seven patients suffering from various metabolic dyscrasias. Total base was determined by electrodialysis in each specimen. Recovery of base from untreated whole tissue appeared to be as high as from muscle which had been ashed or minced before analysis.

The amount of tissue taken for electrodialysis is important. This is illustrated by observations given in Table IX. Two samples of skin were ashed and concentration of total base determined by electrodialysis. These values are assumed to represent 100 per cent recovery. The remainder of each specimen of tissue was sliced into samples that weighed between 0.2 and 0.6 gm. Recovery of base was complete in the small samples and incomplete in the large ones. The optimum amount of tissue is approximately 0.2 gm. The incomplete recoveries in the larger samples of untreated tissue were believed to be caused by poor diffusion of base from the cellular mass. The large quantity of calcium and magnesium present is a further complicating factor. Recovery of these substances is complete only when small amounts are present. Electrodialysis of untreated tissues is not suitable, therefore, for determination of total base in samples which contain pathological deposits of calcium and magnesium.

TABLE IX

Recovery of Tissue Base by Electrodialysis As Function of Size of Sample

Sample No.	Weight of sample	Total base ashed before analysis	Total base untreated before analysis
	gm.	*m.eq. per kg.*	*m.eq. per kg.*
23	0.215	135.3	136.4
	0.290		133.8
	0.430		123.2
	0.620		124.3
24	0.197	130.2	134.5
	0.227		133.0
	0.263		127.1
	0.348		129.0

The electrodialysis apparatus may be used for determination of ammonia in urine or in other biological fluid. It is more laborious than the aeration method described by Van Slyke and Cullen (13) but has one advantage; *i.e.*, small amounts of material may be used. The determination may be made by one of two procedures. Total base may be determined directly and again after ashing. The difference represents volatile or organic base. It is principally ammonia. An alternate procedure is to set up a unit as for a

routine analysis of total base but to substitute a platinum nail for the membrane tube. The current is allowed to flow until the milliameter is constant. This requires no more than 3 hours. The platinum cathode is removed and rinsed and a prepared membrane cathode put in its correct place. Electrodialysis is allowed to proceed as usual. Only non-volatile base is determined by this procedure. The difference between total base and non-volatile base is assumed to be ammonia.

SUMMARY

This communication describes a modified unit for the determination of total base by electrodialysis. Its application is described for the determination of base in serum, blood cells, urine, tissues, and other biological media. Approximately 0.2 ml. or 0.2 gm. of material is adequate. Recovery of total base is complete.

Comparison was made between recovery of base by electrodialysis and by determination of ions individually, by the gravimetric procedure, by the barium iodate method described by Van Slyke and associates, and by the benzidine sulfate method of Hald. Agreement was satisfactory with all of the methods except that described by Hald.

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A STRUCTURAL INTERPRETATION OF THE ACIDITY OF GROUPS ASSOCIATED WITH THE HEMES OF HEMOGLOBIN AND HEMOGLOBIN DERIVATIVES

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Since the discovery by Bohr and coworkers in 1904 of the effect of acidity on the oxygen equilibrium of hemoglobin, there have been a large number of investigations directed towards clarification of the physicochemical relationships of acid groups with the hemes in hemoglobin and its derivatives. It is the purpose of this paper to analyze modern quantitative data, including those for ferrihemoglobin (methemoglobin), in order to throw new light on the general problem, to establish the ionization constant of an acid group in ferrihemoglobin previously unrecognized, and to give a structural interpretation of shifts in the pK of groups caused by changing chemical environment.

Characterization of the Acid Groups by pK Values

In recent papers (1) Taylor and Taylor and Hastings have summarized results of a detailed investigation of the hemoglobin electrode potential in the pH range 5 to 9, obtained with borate and phosphate buffers of ionic strength 0.1 to 0.2. When the potentials measured for solutions containing equal quantities of horse ferrohemoglobin and ferrihemoglobin are plotted against pH, there is obtained a line with slope $\Delta E_0'/\Delta \text{pH}$ of zero at pH 5, curving smoothly to a line of slope -0.06 ($-2.30\ RT/F$) between pH 8 and 9, and the points are satisfactorily represented by the well known theoretical E.M.F.-pH equation with the assumption of an acid group of pK 6.65 for the oxidized form. No evidence

* Contribution No. 746.

was observed in the data Dr. Taylor has kindly put at our disposal for other pK values for either the ferro or the ferri form.

It is of interest to correlate this information about ionization constants of groups connected with the hemes with what is already known about the ionization constants of groups in ferrohemoglobin and ferrihemoglobin. Wyman's analysis (2) of the German and Wyman (3) differential acid-base titration data between ferrohemoglobin and oxyhemoglobin established the existence in ferrohemoglobin of two acid groups of pK 5.25 and 7.81 whose pK values are shifted upon oxygenation to 5.75 and 6.80. Haurowitz (4) showed the existence of an acid group in horse ferrihemoglobin of pK 8.2, a value which has been checked at ionic strength 0.10 for dog hemoglobin spectrophotometrically as 8.10 by Austin and Drabkin (5) and for cow hemoglobin magnetometrically as 8.07 by Coryell, Stitt, and Pauling (6).

There have thus been recognized four acid groups associated with the hemes of ferrohemoglobin or ferrihemoglobin with pK values in the range 5 to 9, each of which is of significance to the physical chemistry of hemoglobin. The electrode potential E_0' of a one-step system at half reduction is commonly expressed by the general equation

$$E_0' = E_0 + \frac{RT}{F}\left[\sum \ln (K_r + \mathrm{H}^+) - \sum \ln (K_o + \mathrm{H}^+)\right] \qquad (1)$$

where K_r is the acid constant of the ferro or reduced form, and K_o that of the ferri or oxidized form. The great majority of the acid groups of the protein parts of hemoglobin compounds has identical values of K_r and K_o and leads to no observable effect of pH on E_0'. If a value of K_o for one acid group accidentally agrees with a value of K_r for another, a similar cancelation of effects occurs. Now the decrease of magnetic susceptibility observed (6) in solutions more acid than pH 6 and the decrease in the dissociation constant of ferrihemoglobin fluoride in acid solutions noted by Lipmann (7) point to the existence of a previously unrecognized heme acid group in ferrihemoglobin with a relatively low pK value, whose ionization affects somewhat the magnetic properties of ferrihemoglobin and the affinity of the iron atom for fluoride ion. The existence of this acid group is made certain by the fact that the values of E'_0 of Taylor are constant between pH 5.0 and 6.0,

which requires that ferrihemoglobin possess an acid group with pK practically identical with that, 5.25, of the known (2) acid group of ferrohemoglobin.

We estimate that a difference in pK values of the two groups greater than 0.2 would have produced an observable effect in the electrode potential data in this pH range, and we assign to the new acid group the approximate pK value 5.3.

A similar cancelation seems to occur in the potentiometric study, within experimental error, between the pK of ferrihemoglobin at 8.1 and that of ferrohemoglobin at 7.81. The effect on E_0' of the ferrihemoglobin acid group with pK 6.65 (change of slope) would tend to mask the small effect (about 18 millivolts extended over 2 pH units) of the real difference in pK of these two acid groups.

It is interesting that oxyhemoglobin and carbonmonoxyhemoglobin have identical pK values, as shown by the work of Hartridge

TABLE I

Heme-Linked Acid Groups in pH Range 4.5 to 9

Hb^+	$pK_1 = 5.3$ *Mo*	*Pi*	$pK_2 = 6.65$ *Si, Mi, Po*	$pK_3 = 8.10$ *So, Mo*	*Pi*
Hb	$pK_1 = 5.25$ *Mi*			$pK_2 = 7.81$ *Si, Mi*	
HbO_2, HbCO	$pK_1 = 5.75$ *Mi, Po*		$pK_2 = 6.80$ *Si, Mi, Po*		

(8) on the oxyhemoglobin-carbonmonoxyhemoglobin equilibrium, which is independent of pH over the range from the point of acid destruction of oxyhemoglobin (about pH 6) to quite alkaline solutions. It will be of considerable importance to determine pK values of such groups associated with the hemes in other hemoglobin derivatives; the case discussed above suggests that only a relatively small number of different categories exist. Russell and Pauling (9) report an additional acid group with pK 9.5 in the covalent ferrihemoglobin-imidazole complex. It seems probable that this group is the imino group in the imidazole ring rather than another acid group of ferrihemoglobin.

The pK values of the known acid groups associated with the hemes of ferrihemoglobin (Hb^+), ferrohemoglobin (Hb), and oxyhemoglobin and carbonmonoxyhemoglobin (HbO_2 and HbCO) are collected in Table I. They are numbered in the order of increasing pK values, and are described by the symbols *S*, *M*, and

P, denoting spectrophotometrically, magnetometrically, and potentiometrically, respectively, and *o* or *i*, denoting operative or inoperative. Since each acid group has a definite effect in any physicochemical equilibrium involving the substance containing it, all are of the class *Po*, but *Pi* is used with brackets where cancelation occurs among these in the ferrohemoglobin-ferrihemoglobin electrode potential.

The two acid groups of ferrohemoglobin have been called the "oxy-labile" groups. Since the acidity of these groups has been shown to be affected by the oxidation to ferrihemoglobin, it is proposed that the more general name *heme-linked groups* be used for them instead. For convenience we designate the various heme-linked acid groups which give rise to pK_1, pK_2, and pK_3 as Groups I, II, and III respectively, and the forms of hemoglobin derivatives predominating in the pH ranges just before neutralization has proceeded half-way as forms I, II, and III. In the case of ferrihemoglobin, the form occurring in alkaline solution with all acid groups neutralized has been recognized as the hydroxide complex, HbOH (6).

Magnetometric Evidence for Value of pK_1 of Ferrihemoglobin

From the data reported in Table III of Coryell, Stitt, and Pauling (6) we have been able to check indirectly the value of pK_1 for ferrihemoglobin given in Table I. The magnetic data were treated assuming the value 5.30 for pK_1 and a calculation made of the effect of the acid form in lowering the observed susceptibility below the asymptotic value given in Fig. 1 of the Coryell, Stitt, and Pauling paper. Their figure has been amended and is presented here as Fig. 1. The broken portions of Curves A and B represent the asymptotic susceptibility which applies to the second form (Hb^+ II). The solid curve falling off in the acid range was calculated for the value 5.30 for pK_1 and the value $12{,}570 \times 10^{-6}$ C.G.S.U. for the molal paramagnetic susceptibility of the most acid form (corresponding to an increase in susceptibility on the loss of the proton of 1500×10^{-6}). The average deviation of the points in the acid range is 110×10^{-6}, comparable to deviations found in the other magnetic studies. This is accordingly a satisfactory treatment of the magnetic susceptibility data for ferrihemoglobin in the pH range from 5 to 12. We

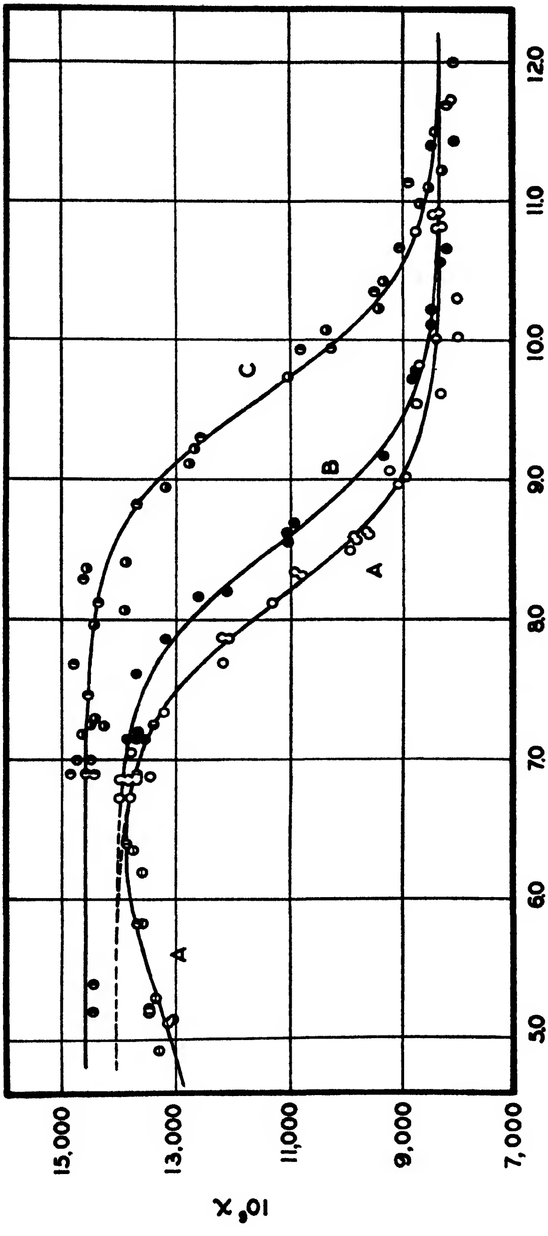


Fig. 1. Dependence of magnetic susceptibility of ferrihemoglobin solutions on pH. Curve A, ionic strength 0.20, $pK_1 = 5.30$, $pK_2 = 8.15$. Curve B, ionic strength 1.3, $pK_2 = 8.56$; Curve C, low and high ionic strength with added fluoride, apparent $pK_3 = 9.62$. Molal susceptibilities ($\times 10^{-6}$ c.g.s.u.) Hb^+I, 12,570; Hb^+II and Hb^+III, 14,070; HbF, 14,610; HbOH, 8340.

estimate that the magnetic determination of the value 5.30 for pK_1 is reliable to about 0.2 unit. The data show that ionization of the second acid group ($pK_2 = 6.65$) causes no change in magnetic properties.

The approximation of two points close to the fluoride Curve C in the acid range (pH $\sim$ 5.3) indicates either that the first acid group of ferrihemoglobin fluoride does not ionize in the range investigated or that it is magnetically inoperative. (The data of Lipmann (7) are not extensive enough for a quantitative treatment of this question.) The inflection at pH 9.6 on this curve is that for replacement of fluoride ion (bonded to the iron atom) by hydroxide ion in 0.34 M fluoride solution.

Structural Interpretation of Acidity of Heme-Linked Acid Groups

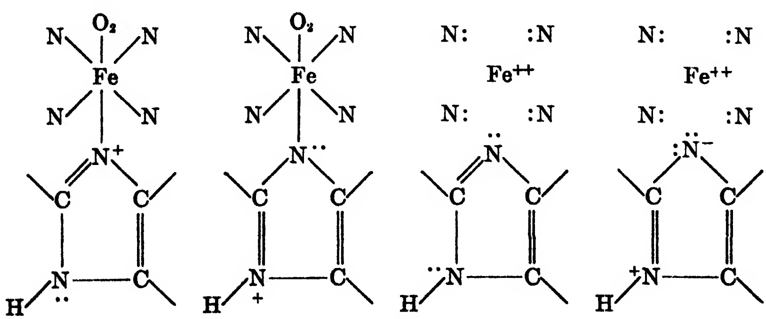
The reduction of ferrihemoglobin to give ferrohemoglobin is accompanied by the loss of one plus charge for the iron atom, and one acid group for the molecule in the range which has been explored (Table I). The formation of ferrihemoglobin fluoride involves bonding the fluoride ion to iron by an ionic bond (6), and the fluoride ion is displaced by the hydroxide ion in alkaline solution. Since reaction with fluoride gives rise to an apparent shift in pK_3 of ferrihemoglobin in accord with the physicochemical requirements of this equilibrium (6) (see Fig. 1), we conclude that the corresponding acid Group III is due to the ferric atom itself. The behavior of a positive ion as an acid group is a well known phenomenon, as for instance in the first step of hydrolysis of the free ferric ion.

It seems reasonable, furthermore, that there are two other acid groups associated with the hemes, for which changes in the bonding of the iron atom give rise to the various values observed for pK_1 and pK_2. Wyman (2) has already concluded from a consideration of the pK values themselves and of the heats of ionization (about 6500 calories per equivalent) of the group that both of these acid groups are due to imidazole groups of histidine residues contained in the globin part of the molecule. We present here structural explanations for the shifts from the values observed in ferrohemoglobin caused by complex formation (with oxygen or carbon monoxide) and by oxidation.

Let us first consider acid Group II. Since the iron atom is

held by essentially ionic bonds in ferrohemoglobin (10) and also in ferrihemoglobin (6), the chief effect of oxidation is to increase the positive formal charge on the iron atom by 1 unit. This is seen to have the effect of decreasing pK_2 by 1.16 units. Assuming that the electrostatic interaction of the iron atom and the proton of the acid group is solely responsible for this change in pK_2, we can estimate roughly the distance between acid Group II and the iron atom from comparison with some dipositive acids such as the salts of the alkyl diamines. The first and second pK values of propylenediamine and butylenediamine differ by 2.04 and 1.54 units, respectively (11). These values must be decreased by log 4 or 0.60 for the symmetry effect, which does not operate between the chemically unlike second and third acid groups of ferrihemoglobin. The observed decrease in pK_2 on oxidation of the iron atom lies between the corrected diamine differences (ΔpK) 1.44 and 0.94. The distance between the nitrogen atoms of the two diammonium salts, assuming extended configuration and the angles and distances given by Pauling (12), are 4.94 and 6.27 Å., respectively. We conclude that acid Group II of ferrohemoglobin and ferrihemoglobin is roughly 5 Å. from the iron atom, about the expected distance between an iron atom near one of the ring nitrogen atoms of a histidine residue and the second nitrogen atom of the ring.

On combination of ferrohemoglobin with oxygen or carbon monoxide, the bonds to the iron atom change from the essentially ionic to the essentially covalent type (10). In order to explain the shift in pK_2 caused by this change ($\Delta pK_2 = -1.01$ units),



(A) (B) (A′) (B′)

HbO_2 Hb

we make the assumption that the iron atom is close to one of the histidine heterocyclic nitrogen atoms. Important structures contributing through resonance to the normal states of the two compounds are shown in the accompanying diagrams. Only the imidazole skeletons of the histidine residues and the 4 nitrogen atoms of the porphyrin group are shown.

The effect of resonance on acid strength may be discussed in the following way (12). If Structure A alone represented the normal state of oxyhemoglobin, the acidity of the NH group of the imidazole ring would be very low, since the structure $-\underset{|}{\ddot{\mathrm{N}}}-\mathrm{H}$ is characteristic of amines (such as dimethylamine) which are basic rather than acidic. If Structure B alone represented the normal state, the group would be rather strongly acidic; the group $=\underset{|}{\mathrm{N}^{+}}-\mathrm{H}$ in the pyridinium cation,[1] for example, has $\mathrm{p}K_{\mathrm{A}} = 5.1$. With resonance between Structures A and B, the group should be somewhat less acidic than the pyridinium cation. This is observed for imidazole derivatives of this type; thus the observed value (13) of pK for the N-methylimidazolium cation is 7.35. For the imidazolium ion itself the observed value of pK, 6.95, becomes 7.25 when corrected by the amount of log 2 to correct for the presence of 2 equivalent ionizable hydrogen atoms; and the imidazolium group in the histidine cation (14) has pK = 6.04, which becomes 6.34 on correction, the change from the imidazolium value being attributable to interaction with the charges in the ionized amino and carboxyl groups of the amino acid. Some variation in substituted imidazoles is to be expected also because of the difference in electronegativity of the attached groups. We would predict on the basis of our postulate about the structure of oxyhemoglobin that $\mathrm{p}K_2$ for this substance should lie in this region, near 7; the observed value, 6.80, is in satisfactory agreement with this prediction.

A qualitative prediction of the effect on $\mathrm{p}K_2$ of removing the oxygen molecule can be made, in the following way. Structures A and B for oxyhemoglobin are seen to be closely similar (they are equivalent in the imidazolium ion), and hence they contribute

[1] The Kekulé-like resonance in this substance is not expected to affect the acidity very greatly.

nearly equally to the normal state of the molecule. The decrease from large contribution of a structure of the type of Structure B (pyridinium ion) to a contribution of about 50 per cent is accompanied by an increase in pK by about 2 units. Now in ferrohemoglobin itself Structure B′ makes a still smaller contribution than 50 per cent, because this structure, with separated electric charges, is less stable than Structure A′, in which the nitrogen atoms have their normal covalence; hence it is predicted with certainty that *the change of bond type for the iron atom accompanying removal of the oxygen molecule must be accompanied by a decrease in the acidity of the attached imidazole group.* A quantitative prediction of the magnitude of the expected change in pK_2 from oxyhemoglobin to ferrohemoglobin cannot be made at present; but the observed change from 6.80 to 7.81 is reasonable, in the light of the above discussion.

In explaining the changes in pK_1 recorded in Table I we make use of the suggestion of Conant (15) that a second imidazole ring of a histidine residue lies near the opposite side of the porphyrin ring from the one responsible for acid Group II. We assume that the 3-nitrogen atom of this ring is restrained by the configuration of the hemoglobin molecule to a relatively unfavorable position for electrostatic coordination with the iron atom, so that a proton can be added, breaking the bond to the iron atom, at high enough acidity. These assumptions explain the occurrence of the low pK_1 value 5.25 in ferrohemoglobin for the imidazolium structure postulated for acid Group I. The coordination of the iron atom with an oxygen molecule on the same side of the porphyrin ring would be expected to prevent the interaction with the iron atom and thus to make the imidazolium group show more nearly the same pK value as in histidine itself (pK = 6.04).

On oxidation of the iron to the ferric form, the pK_1 value would at first sight be expected to be lowered, as is the pK_2 value; instead, no appreciable change is observed. If, however, after addition of a proton to the imidazole group a water molecule coordinates (through dipole attraction) with the iron atom in the ferric state more strongly than with it in the ferrous state, the corresponding extra stabilization of the acid form by the water molecule will tend to offset the expected decrease in pK_1 when the iron atom is oxidized. The cancelation of these effects seems to be complete.

It is noteworthy that physicochemical analysis offers the main method for studying the nature of the binding of the prosthetic group to the protein in hemoglobin. Without doubt, theories which stand the test of further experimental investigation of this substance will be of general value in the study of other heme pigments. The spectroscopic study of the acid groups of ferricytochrome C by Theorell and Åkesson (16) together with the as yet unpublished new magnetic studies carried out in these laboratories by Theorell illustrates this point.

We are indebted to Dr. J. F. Taylor of Harvard University for making the results of his experiments available to us before their publication in detail.

SUMMARY

The existence of an acid group interacting with the heme in ferrihemoglobin (methemoglobin) with the pK value 5.3 has been established from the consideration of electrode potential data and magnetic susceptibility data. The magnetic susceptibility of ferrihemoglobin over the pH range 5 to 12 has now been completely correlated with heme-linked acid group ionizations.

A table is presented giving the spectroscopic and magnetic characteristics of the three known heme-linked acid groups of ferrihemoglobin, the two of ferrohemoglobin, and the two of oxyhemoglobin and carbonmonoxyhemoglobin occurring in the pH range 4.5 to 9.5. An explanation is offered for the fact that only one effective pK value is observed in studies of the ferrohemoglobin-ferrihemoglobin electrode potential.

Structural interpretations of the acidity of the heme-linked acid groups in hemoglobin and the changes in acidity caused by oxygenation and oxidation are given. It is postulated that acid Group I is a histidine imidazolium ion in poor position for electrostatic coordination of the basic form with the iron atom, and that acid Group II is the imino group of a histidine residue (on the opposite side of the porphyrin ring from acid Group I) whose 3-nitrogen atom is strongly coordinated by either an essentially ionic or an essentially covalent bond with the iron atom. Acid Group III of ferrihemoglobin is the iron atom itself, which may add hydroxide ion, or a water molecule coordinated to the iron atom, which may lose a proton.

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A COMPARISON OF THE OPTICAL FORMS OF GLUTAMIC ACID FROM NORMAL AND CANCEROUS TISSUE

Sirs:

The work of Kögl and Erxleben[1] has indicated that a large proportion of the glutamic acid obtained by hydrochloric acid hydrolysis of cancerous tissues appears as the *d*(−) form. It

	Jensen sarcoma rats						Liver of normal rats		
	Tumor			Liver					
	Weight	$[\alpha]_D^{20}$ in 9 per cent HCl	Per cent *d* form	Weight	$[\alpha]_D^{20}$ in 9 per cent HCl	Per cent *d* form	Weight	$[\alpha]_D^{20}$ in 9 per cent HCl	Per cent *d* form
	gm.	*degrees*		*gm.*	*degrees*		*gm.*	*degrees*	
Fresh	149			154			211		
Dried	20			43			45		
Crude glutamic acid hydrochloride	2.6			2.1			5.47		
Recrystallized 3 times	0.774	+31.4		0.929	+31.7		2.40	+31.5	
Concentrated mother liquors of above	1.376	+12.8					0.858	+29.8	
Recrystallized once	0.478	+15.6		0.588	+23.9		0.649	+31.1	
Recrystallized twice	0.251	+17.2	23.0	0.384	+27.1	7.1			
Crystals from mother liquors of above	0.262	+14.9	26.3	0.360	+17.0	23.0	0.538	+2.4	
Recrystallized again							0.310	+1.88	47.0
Calculated for total crude glutamic acid			2.6			3.9			2.4
Pure *l*(+)-glutamic acid hydrochloride		+31.5							

seemed desirable to make a study of the Jensen rat sarcoma. Furthermore, the normal livers of rats bearing this tumor as well as the livers from normal rats were used for comparison. The dried

[1] Kögl, F., and Erxleben, H., *Z. physiol. Chem.*, **258**, 57 (1939).

tissues were hydrolyzed by gentle boiling with 20 per cent hydrochloric acid for 30 hours. Glutamic acid was isolated from the hydrolysate by hydrochloric acid precipitation, with barium salt treatment of the filtrate. The crude glutamic acid hydrochloride was recrystallized from 20 per cent hydrochloric acid to constant specific rotation. The mother liquors from the crude glutamic acid hydrochloride were concentrated under reduced pressure at a temperature under 45°. The crystals obtained were recrystallized from 20 per cent hydrochloric acid to constant rotation. The presence of cystine as a cause of the lowered rotations was ruled out each time by a nitroprusside test after reduction with sodium cyanide.

The results indicate that, whereas $l(+)$-glutamic acid is the principal constituent of the glutamic acid fraction from tumor and from normal liver tissues, it seems probable that a small amount of $d(-)$-glutamic acid is also present in the hydrolysate of *normal* as well as tumor tissue. These findings suggest the possibility of a slight racemization during hydrolysis of the protein.

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THE RELATION OF THE FOLLICLE-STIMULATING ACTIVITY OF FRESH PITUITARY TISSUE TO THE ACTION OF THE ENZYMES CONTAINED IN THE TISSUE

Sirs:

It has been shown that the follicle-stimulating hormone contained in aqueous extracts of sheep pituitary powder is not inactivated by digestion with commercial or crystalline trypsin, while the luteinizing activity is destroyed by trypsin.[1] This finding suggested that under the proper conditions the proteolytic enzyme (probably cathepsin) of fresh pituitary tissue might inactivate the luteinizing hormone without the destruction of the follicle-stimulating activity. This assumption was substantiated by preliminary experiments with fresh tissue treated according to the procedure given in this communication.

Fresh sheep pituitaries were ground finely while still frozen and thoroughly mixed with 2 volumes of distilled water. The suspension was adjusted to pH 4.2 by the addition of acetic acid, followed by incubation at 37° for 8 hours. The digest was centrifuged and both the residue and the clear supernatant liquid were tested by subcutaneous administration over 4.5 days to normal rats 21 days old.

The ovaries from the rats that received the supernatant liquid in doses of 1 and 2.5 gm. equivalents of fresh tissue had an average weight of 40 and 65 mg. respectively and contained follicles only, while the residue from the digest was inactive. The suspension of pituitary tissue that was kept in the cold at pH 4.2 and used as control produced ovaries that contained many corpora lutea.

When the clear supernatant extract which contained the follicle-stimulating activity was separated from the insoluble residue of the digest and added to 4 volumes of acetone, a stringy substance precipitated immediately. This substance did not contain gonad-

[1] McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, **126**, 361 (1938).

otropic activity and was removed readily from the cloudy acetone solution. The follicle-stimulating activity which remained in the acetone solution was recovered and was found to be associated with relatively little solid material.

The indications are that it may be possible to use this inexpensive and simple procedure in the preparation of follicle-stimulating material of low solid content, and that further information in regard to the chemistry of the hormone may be gained from material obtained by this method.

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INCREASING THE RATE OF EXCRETION OF SELENIUM FROM SELENIZED ANIMALS BY THE ADMINISTRATION OF *p*-BROMOBENZENE*

Sirs:

It has been shown by Stekol[1] that *p*-bromobenzene administered to animals is detoxified by conjugation with tissue cystine and methionine and excreted in the urine as *p*-bromophenylmercapturic acid. Since selenium occurs in the body proteins of animals fed seleniferous diets, and since it is possible that it may

Before C_6H_5Br	Days after administration of C_6H_5Br* began					
	1	4	7	13	20	54
	Se in blood, p.p.m.					
3.33†	1.46	1.33	0.16	0.02	0.000	2.4
2.53‡	0.19	0.16	0.16	0.000	0.000	2.0

Se in urine, p.p.m.

	1	2	3	4	5	6	7	9	11	12	14	16	20
0.393†	2.66	1.12	1.80	1.04	1.00	2.20	1.20	1.4		1.0	1.6		2.0
0.705‡	0.461	0.96	1.92	0.58	0.90	1.80	1.10	0.8	2.4	1.6	1.2	4.0	1.5

* Daily dosage per head 1st to 4th day, 10 cc., 5th to 8th day, 5 cc., 9th to 14th day, 2.5 cc.

† Steer 95. ‡ Steer 96.

replace sulfur in amino acids, it became of interest to determine whether or not selenium would appear in *p*-bromophenylmercapturic acid isolated from the urine of selenized animals.

Experiments were conducted on selenized dogs which had been fed a diet containing 10 p.p.m. of selenium in the form of seleniferous corn and on steers which were grazing on a seleniferous range.

* Approved for publication by the Director of the South Dakota Agricultural Experiment Station.

[1] Stekol, J. A., *J. Biol. Chem.*, **117**, 147 (1937).

During the course of the work with the dogs it was observed that the rate of selenium excretion was greatly increased by the administration of the *p*-bromobenzene. This observation led us to administer the compound (for 14 days) to "alkalied" steers which had been grazing on seleniferous vegetation for 5 months and showed typical symptoms of selenium poisoning. The steers continued on the same feed during treatment. Results on the two most severely selenized steers are shown in the table.

Blood and urine selenium levels of other selenized steers showed similar trends following the administration of *p*-bromobenzene. No selenium could be detected in protein-free filtrates of blood from these steers, and the total selenium content of the blood was accounted for by analysis of the blood protein precipitate. 40 days after the termination of the treatment the selenium levels in the blood of the steers had risen almost to the initial levels. The *p*-bromobenzene appears to aid the organism in excreting the selenium by taking it from the proteins of the blood and tissues, possibly as selenium-cystine and selenium-methionine which are conjugated with the *p*-bromobenzene and excreted as the mercapturic acid. *p*-Bromobenzene has recently been used with excellent results in the treatment of a human case of selenium poisoning.[2]

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[2] Personal communication, Dr. R. E. Lemley, Rapid City, South Dakota.

THE COMPOSITION OF UNSAPONIFIABLE LIPIDS*

Sirs:

In the course of an investigation of lipid metabolism in the brain and other tissues the unsaponifiable lipids were isolated from the carcass (less brain, liver, and intestinal tract) of rats in which the body water had been enriched with deuterium for 4 to 7 days. The deuterium content of this fraction was surprisingly high, exceeding in most rats that found in any organ studied (brain, liver, and intestine). Hence the portions of unsaponifiable material of the carcass remaining after deuterium analysis were pooled from four adult rats and thirteen 30 and 40 day-old rats (1298 mg., 0.25 atom per cent deuterium). Cholesterol was removed by precipitation with digitonin (370 mg., 0.19 atom per cent deuterium). The cholesterol-free unsaponifiable fraction (B) was extracted with petroleum ether from the filtrate after addition of alkali (583 mg., 0.30 atom per cent deuterium). Fatty acids which had not been removed in the original isolation were extracted after acidification (189 mg., 0.14 atom per cent deuterium). From 444 mg. of unsaponifiable Fraction B hydroxy compounds (alcohol fraction) were separated as the half esters of succinic acid, according to Dutcher and Wintersteiner[1] (126 mg., 0.43 atom per cent deuterium). From 244 mg. of the non-alcoholic fraction keto compounds were separated with Girard's[2] reagent (159 mg., 0.22 atom per cent deuterium). The remaining (hydrocarbon ?) fraction (33 mg.) contained 0.24 atom per cent deuterium. The losses which occurred during the fractionation make an exact balance sheet impossible.

The findings which we wish to emphasize are first, the proximity

* This investigation was supported by a grant from the Friedsam Fund donated to the Division of Child Neurology, Neurological Institute, New York.

[1] Dutcher, J. D., and Wintersteiner, O., *J. Am. Chem. Soc.*, **61**, 1992 (1939).

[2] Girard, A., and Sandulesco, G., *Helv. chim. acta*, **19**, 1095 (1936).

of deuterium concentrations in the cholesterol, keto, and hydrocarbon fractions, suggesting a close metabolic relation among them, and, secondly, the high isotope content of the alcohol fraction which contained approximately twice as much deuterium as the other unsaponifiable fractions and more with few exceptions than any fatty acid fraction isolated from any tissue during this work. The latter finding suggests that the compounds comprising the alcohol fraction are intermediates in the synthesis of fatty acids. An alternate interpretation would be that they are formed in the dismutation of aldehydes (plasmal[3]) *in vivo* or *in vitro* during alkaline hydrolysis. The investigation of the unsaponifiable fraction with deuterium as an indicator is being continued, with particular attention to the possible rôle of aldehydes in its genesis and in the synthesis of fatty acids.

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[3] Feulgen, R., and Bersin, T., *Z. physiol. Chem.*, **260**, 217 (1939).

OBSERVATIONS ON THE FACTOR CURATIVE OF NUTRITIONAL ACHROMOTRICHIA

Sirs:

In rats kept on a basal diet deficient in vitamin B and supplemented with vitamin B_1, riboflavin, and vitamin B_6 cutaneous lesions may appear which, in several instances, may become severe and even generalized.[1] In the same group of rats fur alterations also can be observed in the form of depigmentation ("graying") in piebald or black animals and of "rusting" in albino animals. These fur changes, which appear to be identical with those seen by several authors[2-5] in rats kept under similar nutritional conditions, were considered by Morgan *et al.*[3] and Lunde and Kringstad[4] to be due to lack of one of the filtrate factors. Recently Oleson *et al.*[5] reported that according to their experiments the dietary factor which prevents nutritional depigmentation of the fur (achromotrichia) in rats appears to be "distinct from all factors of the vitamin B complex which have thus far been identified and associated with specific function in the nutrition of the rat."

In our curative experiments, when concentrates of pantothenic acid with a degree of purification up to 40 to 50 per cent were added to the vitamin B-free diet containing vitamin B_1, riboflavin, and vitamin B_6, not only did the cutaneous lesions show distinct regression[6] but the blackening of the fur also became definitely

[1] György, P., *J. Am. Chem. Soc.*, **60**, 983 (1938). György, P., and Eckardt, R. E., *Nature*, **144**, 512 (1939).

[2] Bakke, A., Aschehoug, V., and Zbinden, C., *Compt. rend Acad.*, **191**, 1157 (1930); *Chem. Abst.*, **25**, 989 (1931).

[3] Morgan, A. F., Cook, B. B., and Davison, H. G., *J. Nutrition*, **15**, 27 (1938).

[4] Lunde, G., and Kringstad, H., *Z. physiol. Chem.*, **257**, 201 (1939).

[5] Oleson, J. J., Elvehjem, C. A., and Hart, E. B., *Proc. Soc. Exp. Biol. and Med.*, **42**, 283 (1939).

[6] György, P., Poling, C. E., and Subbarow, Y., *Proc. Soc. Exp. Biol. and Med.*, in press.

evident. Administration of alkaline concentrates which had been autoclaved had no effect on either of these pathologic manifestations.

The first sign of blackening is a bluish discoloration of the skin, which is due probably to the first growth of normally pigmented hair shafts in the epidermis. Then the depigmented brown, gray, or white hair falls out and black fur appears.

The curvative doses of the concentrates varied between 75 and 200 γ in terms of pantothenic acid.

It should be pointed out that under the influence of concentrates of pantothenic acid the fur, as a rule, does not become completely black and depigmented areas often persist. In several rats the blackening progressively increased with larger doses.

These findings explain the inability of Oleson *et al.*[5] to prevent the appearance of achromotrichia, on the one hand, and the accomplishment of a delay in its appearance, on the other.

In summary, concentrates of pantothenic acid, with a purification up to 40 to 50 per cent, appear to contain one factor but not the only factor concerned in the cure of nutritional achromotrichia in rats.

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FRACTIONATION OF THE BACTERICIDAL AGENT FROM CULTURES OF A SOIL BACILLUS

Sirs:

The protein-free bactericidal material isolated from a spore-bearing soil bacillus[1] has been further purified and three crystalline preparations highly bactericidal for Gram-positive microorganisms have been obtained. Two acid substances are isolated by solution of the crude material in alcohol, precipitation with 15 volumes of ether, and fractional crystallization of the dried precipitate from hot absolute alcohol. The third substance, which we have named gramicidin, is concentrated by repeatedly recovering the fraction which remains soluble in alcohol on the addition of 15 volumes of ether but is insoluble in absolute ether. Crystallization is effected by extraction with a mixture of equal volumes of acetone and ether, evaporating the extracts, and cooling a solution of the residue in boiling acetone. From 100 gm. of crude material there were isolated in all about 60 gm. of acid of which at least the larger part could be separated as one or the other of the two crystalline acids and approximately 10 to 15 gm. of crystalline gramicidin. It appears probable that other active substances may be present. Residual fractions of alcohol-insoluble material and ether-soluble matter (fatty acid) are inactive.

Of the acids the one least soluble in alcohol, designated graminic acid, crystallizes as colorless hexagonal prisms or platelets. The melting point is 232–234° (uncorrected). The other fraction, larger in amount, and more soluble in alcohol, we have called gramidinic acid. It crystallizes in clusters of microscopic needles, melting with decomposition at about 230°.

Gramicidin crystallizes from acetone as characteristic spear-shaped, colorless platelets. These show a melting point of 228–230°. The substance is even more sparingly soluble than the acid

[1] Dubos, R. J., and Cattaneo, C., *J. Exp. Med.*, 70, 249 (1939).

compounds in water and more soluble in alcohol and acetone. The specific rotations in 95 per cent alcohol solution are approximately $[\alpha]_D^{25} = -115°$ (graminic acid), $-100°$ (gramidinic acid), and $+5°$ (gramicidin).

All three substances are very effective in killing Gram-positive microorganisms. An actively growing Type I pneumococcus culture is sterilized within 1 hour by the addition of 10 micrograms of the acids or by 5 micrograms of gramicidin. 5, 2, or even 1 microgram of gramicidin when administered by the intraperitoneal route will protect a large percentage of mice infected intraperitoneally with 10,000 fatal doses of virulent Type I pneumococci. Graminic and gramidinic acids in even much larger quantities, on the other hand, will not prevent the death of the animal. Similar results have been obtained with other Gram-positive microorganisms, while Gram-negative organisms are unaffected either *in vitro* or *in vivo*.

Intraperitoneally 0.3 mg. of gramicidin will kill mice and smaller quantities will cause marked toxic reactions. It is evident from the few experiments already completed that the substance must be regarded as highly toxic. The non-protective crystalline acids appear to be considerably less toxic.

Since the crystalline substances appear to be closely related, it is hoped that a chemical study of the differences between the protective and non-protective substances may reveal some of the factors which make it possible for a bactericidal agent to be effective *in vivo*.

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CHEMICAL PROPERTIES OF BACTERICIDAL SUBSTANCES ISOLATED FROM CULTURES OF A SOIL BACILLUS

Sirs:

This is an account of the preliminary chemical investigation of the crystalline bactericidal substances described in the preceding communication. Gramicidin, which has been most investigated, contains 62.7 per cent C, 7.5 per cent H, 13.9 per cent N. The molecular weight as determined in camphor is about 1400. Since with substances of this complexity the accuracy of the determinations is limited, an empirical formula will be presented only with certain reservations. The data can be represented by the formula $C_{74}H_{106}N_{14}O_{14}$ or formulæ differing from it by 1 or 2 atoms of carbon or hydrogen or by the difference C_5H_7NO.

Gramicidin contains neither free basic nor acidic groups. Upon acid hydrolysis a quantity represented by the above formula liberates (*a*) a total of 11 equivalents of non-volatile acid, (*b*) 11 equivalents of basic groups, (*c*) 10 moles of α-amino acid (determined by the Van Slyke ninhydrin procedure), (*d*) 2.4 moles of tryptophane (determined by colorimetric dimethylaminobenzaldehyde reaction), (*e*) a fraction of a mole of a 14-16-carbon saturated aliphatic acid. Although tryptophane is fairly stable in acid when pure or in the absence of carbohydrates or aldehydes, some destruction occurs and an error may result especially in (*b*) and (*d*). The substance contains neither methoxyl nor acetyl groups. Histidine, arginine, tyrosine, and ammonia have not been found in the hydrolysate.

The above results suggest that gramicidin is essentially a polypeptide containing 10 molecules of α-amino acids of which 2 or 3 are tryptophane residues. These and the aliphatic acid account for about 85 to 90 per cent of the weight of the substance. The nature of the particular amino acids and the remaining constituents of the molecule is being investigated.

Graminic acid and gramidinic acid are similarly built up from

amino acids but contain one free carboxyl group per molecule. They appear to contain only one tryptophane group per molecule and furthermore each of them contains the amino acid tyrosine. Graminic acid (C 58.9, H 7.0, N 14.0) with a molecular weight of about 900 can be represented by the formula $C_{44}H_{63}N_9O_{11}$ (with the same alternatives as for the formula given before). Gramidinic acid appears to have a molecular weight of approximately 1000. Completely reliable analyses have not as yet been obtained with this last compound.

Through the cooperation of Dr. Fritz Lipmann of Cornell University Medical College indications have been obtained through the use of Krebs' *d*-amino acid oxidase[1] that acid hydrolysates of gramicidin and of gramidinic acid have nearly one-half their α-amino acids in the form of *d*- (the so called unnatural) amino acids. Provided racemization has not occurred to an unusually great extent during hydrolysis, therefore, it seems possible to conclude that these bactericidal polypeptides contain a large proportion of *d*-amino acids. This possibility may have significance for the biological activity of the substances.

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[1] Lipmann, F., Behrens, O. K., Kabat, E. A., and Burk, D., *Science*, **91**, 21 (1940).

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